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An extracellular drug binding site of the potassium channels THIK-1 and THIK-2

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Abstract

Background: THIK-1, THIK-2 and TREK-1 and all belong to family of two-pore-domain potassium channel (K_{2P} channels). THIK-2 was until recently regarded as 'silent' potassium channels. 3-isobutyl-1-methylxanthine (IBMX) is normally used as inhibitor of phosphodiesterase, resulting in an increase of cAMP levels in the cytosol.

Aims: Identification of an extracellular drug binding site on THIK-1 and THIK-2.

Methods: Whole cell recording patch clamp measurements in mammalian cells was used to analyze K_{2P} channels mentioned above. Chemicals such as IBMX, forskolin and cAMP were used intracellularly (via the pipette solution) and/or extracellularly (via the bath solution). To identify the binding site of IBMX on THIK-1 we mutated all amino acids of the helical cap one by one and screened for changes in IBMX sensitivity of the channels. To analyze the surface expression of the channel we used HA-tagged THIK-2 and thus quantified the copy number of the channels at the cell membrane using an antibody-based assay.

Results: We found that IBMX can rapidly inhibit both the inward and outward currents carried by THIK-1 channels; the IC_{50} of this effect was about 120 µM. The application of H89 (PKA inhibitor) and forskolin (PKA activator) did not modify the effects of IBMX on the channel. Application of 100 µM intracellular cAMP almost completely inhibited TREK-1 current but not THIK-1 current, indicating that the effect of IBMX in THIK-1 is not mediated by cAMP. Finally, we found that IBMX blocks THIK-1 currents only if it is applied extracellularly. By mutating all of the helical cap amino acids, we found that the arginine to alanine mutant of THIK-1 (THIK-1R92A) had a lower sensitivity to IBMX. Mutation of the arginine at position 92 to glutamate or glutamine reduced the sensitivity to IBMX even further. R92 is localized to the linker region between cap helix 2 (C2) and the pore helix (P1). Part of the linker region is not visible in the crystal structures. R92 is at the end of the unstructured region.

Compared to THIK-1, the 'silent' channel THIK-2 has an additional domain at its N-terminus (residues 6-24) which contains a putative retention signal (RRR). Removal of this additional domain (mutant THIK-$2^{\Delta 6\text{-}24}$) or mutation of the RRR motif to AAA (THIK-2AAA mutant) gave rise to a measurable potassium current. Furthermore, the surface expression of the reporter protein CD74 containing the AAA mutated N-terminus of THIK-2 was more than threefold larger than the analogous reporter protein containing the wild type N-terminus of THIK-2 (RRR). These data indicate that the ER retention/retrieval signal RRR can prevent the THIK-2 export to the cell membrane, leading to the silence of the channel. In addition, we found that THIK-2 currents can also be blocked by application of IBMX from the extracellular side.

Conclusions: IMBX can block TREK-1 channels though the PKA pathway, it also can bind to the extracellular side of THIK-1 or THIK-2, leading to a direct block of the channels. This describes a novel effect of IBXM on K_{2P} channels. The IC50 of the direct effect of IBMX on THIK-1 channels was about 120 µM. Our results suggest that arginine 92 of THIK-1 and the C2-P1 linker region of K_{2P} channels play an important role in the binding of IBMX, and perhaps other more potent drugs, to the channel.

Zusammenfassung

Hintergrund: THIK-1, THIK-2 und TREK-1 gehören zur Familie der Zwei-poren-domänen Kaliumkanäle (K_{2P} Kanäle). THIK-2 wurde bis vor kurzem als inaktiver Kaliumkanal angesehen. 3-isobutyl-1-methylxanthin (IBMX) wird normalerweise als Phosphordiesterasehemmer verwendet, der die intrazelluläre c-AMP Konzentration erhöht.

Ziel der Arbeit: Identifizierung einer extrazellulären Bindungsstelle am THIK-1 und THIK-2.

Methoden: Ganzzellstrommessungen an Säugetierzellen wurden durchgeführt um die obigen K_{2P} Kanäle zu untersuchen. Chemikalien wie IBMX, Forskolin und c-AMP wurden intrazellulär über die Pipette und/oder extrazellulär über die Badlösung zugegeben. Um die IBMX Bindungsstelle am THIK-1 zu finden wurden alle Aminosäuren der äußeren Helices einzeln ausgetauscht und die Stärke des IBMX Blocks ermittelt. Um die Oberflächenexpression des Kanals zu bestimmen, wurde ein HA-markierter THIK-2 Kanal verwendet und mittels eines antikörperbasierten Verfahrens dessen Oberfächenkonzentration quantifiziert.

Ergebnisse: Wir haben gemessen, dass 1 mM IBMX die Einwärts- und Auswärtsströme von THIK-1 sehr schnell blockiert. Die Zugabe von H89, einem PKA-Inhibitor, und von Forskolin, einem PKA-Aktivator veränderte den Effekt von IBMX auf den Kanal nicht.

Die intrazelluläre Zugabe von 100 µM c-AMP blockierte den TREK-1 Strom fast vollständig, beeinflusste jedoch nicht den THIK-1 Strom, woraus man schließen kann, dass IBMX nicht über eine Erhöhung der c-AMP Konzentration auf den THIK-1 wirkt. Außerdem beobachteten wir, dass IBMX die THIK-1 Ströme nur blockiert, wenn es von der extrazellulären Seite zugegeben wird. Eine Mutationen in der extrazellulären Region von THIK-1 and Position 92 (THIK-1R92A) bewirkte eine Abnahme der Affinität der Binding von IBMX an den Kanal. Eine Mutation dieser Aminosäure (R92) zu Glutamin oder Glutaminsäure verringerte die Affinität noch stärker. R92 liegt in der Verbindungsschleife zwischen der Helix 2 und der Porenhelix des Kanals.

Diese Region ist teilweise nicht in der Kristallstruktur sichtbar; R92 liegt am Ende dieser unstrukturierten Region.

Im Vergleich zum THIK-1 besitzt der THIK-2 einen zusätzliches Segment am N-Terminus (Aminosäure 6 - 24) welches ein potentielles Retentionssignal (RRR) enthält. Durch Herausschneiden dieses Segments (THIK-$2^{\Delta 6\text{-}24}$) oder Mutation von RRR nach AAA (THIK-2AAA) konnten THIK-2 Mutanten erzeugt werden, die einen messbaren Strom zeigten. Darüber hinaus konnte gezeigt werden dass die Oberflächenexpression eines Reporterproteins (CD74), das mit dem modifizierten N-Terminus von THIK-2AAA fusioniert wurde, mehr als dreimal so hoch war als unter Kontrollbedingungen (Fusion mit dem nicht modifizierten N-Terminus von THIK-2. Daraus läßt sich schließen, dass das ER Retentionssignal RRR den Transport von THIK-2 zur Zellmembran verhindert und zu einem elektrisch inaktiven Kanal führt. Außerdem fanden wir heraus, dass THIK-2 Ströme ebenfalls durch IBMX von der extrazellulären Seite blockiert werden können.

Schlußfolgerungen: IBMX blockiert TREK-1 Kanäle über die PKA Phosphorilierung. IBMX bindet auch an der extrazellulären Seite von THIK-1 und THIK-2 und führt zu einem direkten Block. Das ist eine neue bisher nicht beschriebene Wirkung von IBMX auf K_{2P} Kanäle. Die Ergebnisse dieser Arbeit deuten darauf hin, daß der C2-P1 Linker und insbesondere das Arginin an der Stelle 92 beim THIK-1 an der IBMX Bindung beteiligt sind.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
cAMP	Adenosine 3′,5′-cyclic monophosphate
CaCl2	Calcium chloride
CHO	Chinese hamster overay
EGTA	Ethylene Glycol Tetraacetic Acid
FBS	fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish-peroxidase
IBMX	3-isobutyl-1-methylxanthine
K_2AT	Adenosine 5′-triphosphate dipotassium salt dihydrate
KCl	potassium chloride
K-Glutamate	L-Glutamic acid monopotassium salt monohydrate
KOH	potassium hydroxide
MgCl2	Magnesium chloride
ms	milliseconds
Na_2GTP	Guanosine 5′-triphosphate sodium salt hydrate
NaCl	Sodium chloride
NaH2PO4	Sodium phosphate monobasic
NaOH	sodium hydroxide
P_o	Open probability
pS	picosiemens
TMD	transmembrane domain
TPA	tetrapentylammonium

1: Introduction

1.1 Ion channels

Ion channels are pore-forming proteins which are highly important and which contribute significantly to cell physiological processes including the regulation of membrane potential, shaping action potential and other electric signals. There are two significant features of ion channels (Alberts *et al.*, 2007):

1. Transport efficiency. Up to 100 million ions can pass through one open channel per second;

2. The transport of ion passing through ion channels is always passive, driven by electrochemical gradients (downhill). There are no active transport or co-transport mechanisms.

3. Selectivity. Normally one ion channel only can conduct one kind of ion.

Until now, there are more than 300 types of ion channels in all biological cells described which display different characteristics and functions (Gabashvili *et al.*, 2007; Kew and Davies, 2010). Classes of ion channels can be distinguished on the basis of electrophysiology, pharmacology, extracellular agonists, intracellular messengers, and sequence homology. Depending on the type of ions that are conducted by the channel there are potassium channels, calcium channels, sodium channels, chloride channels, proton channels and non-selective cation channels.

As ion channels have important roles in physiological processes, multiple diseases, called channelopathies, result from their dysfunction. Channelopathies include autosomal dominant nocturnal frontal lobe epilepsy, achromatopsia 2 (color blindness), episodic ataxia with myokymia, Long-QT syndrome, persistent hyperinsulinemic hypoglycemia of infancy (PHHI), bartter syndrome type III, pseudohypoaldosteronism type 1 and so on(Hübner and Jentsch, 2002; Zaydman *et al.*, 2012). Those pathological conditions emphasize that ion channels play vital roles in physiological processes. Therefore studying ion channels function and regulation is of great importance.

1.2 Potassium channels

Potassium channels are potassium selective, pore-forming proteins that allow potassium ions to pass across the plasma membrane but block other ions, especially sodium ions. They are widely distributed in organism and can be found in most cell types. The major functions of potassium channels are described by two characteristics(Coetzee *et al.*, 1999):

1. Conduct potassium ions pass through membrane down their electrochemical gradient. This process is selective and rapid;

2. Regulate resting potential, action potential and (indirectly) other cellular process such as secretion of hormones.

In generally there are four major types of potassium channels: Two pore domain potassium channel (also called leak K^+ channel or tandem pore domain potassium channel), calcium-activated potassium channel (K_{Ca} channel), inwardly rectifying potassium channel (K_{ir} channel) and voltage-gated potassium channel (K_v channel). Potassium channels can also be classified into three groups dependent on their topological structures: two, four or six transmembrane domains (TMD, Figure 1.1) (Zhong *et al.*, 2013).

2TMD channel: 2TMD channels are inward rectifier potassium channels composed of one pore (P) domain and two TMDs (M1-M2). 2TMD channels include K_{ir} channel and K_{ATP} channel. However, some people classify K_{ATP} channel as a K_{ir} subclass ($K_{ir}6.x$ channel). The K_{ir} channel family is composed by seven subclasses named $K_{ir}1$ –$K_{ir}7$. These channels have multiple functions and vary across cell types. In general, all 2TMD channels contribute to the resting membrane potential. More specifically, $K_{ir}6$ (K_{ATP}) channel expressed in pancreatic can regulate insulin release. $K_{ir}3$ or G protein-coupled inwardly-rectifying potassium channels (GIRKs) expressed in neurons and cardiomyocytes can be activated by neurotransmitters leading to a hyperpolarization of the cell or a decreased heart rate. K_{ir} channels also work on endothelial cells, reuptake of potassium back into the body in kidneys (Nichols and Lopatin, 1997; Hille, 2001). Multiple diseases are related to K_{ir} channels, such as Bartter syndrome (renal salt loss, hypokalemic alkalosis, Long-QT syndrome with

dysmorphic features (Andersen syndrome) and Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Hübner and Jentsch, 2002; Zaydman *et al.*, 2012).

4TMD channel: 4TMD channels or tandem pore domain potassium channels are special channels compared to other potassium channels because each subunit contains two pore-formingn domains. 4TMD channels are active as dimers. Until now, 4TMD channels have five subclasses including tandem of pore domains in a weak inward rectifying K^+ channel (TWIK), TWIK-related K^+ channel (TREK), TWIK-related acid-sensitive K^+ channel (TASK), TWIK-related alkaline pH-activated K^+ channel (TALK) and tandem pore domain halothane-inhibited K^+ channel (THIK). TWIK-related spinal cord K^+ channel is also 4TMD channel and is activated by calcium ions. This part will be discussed in the next section.

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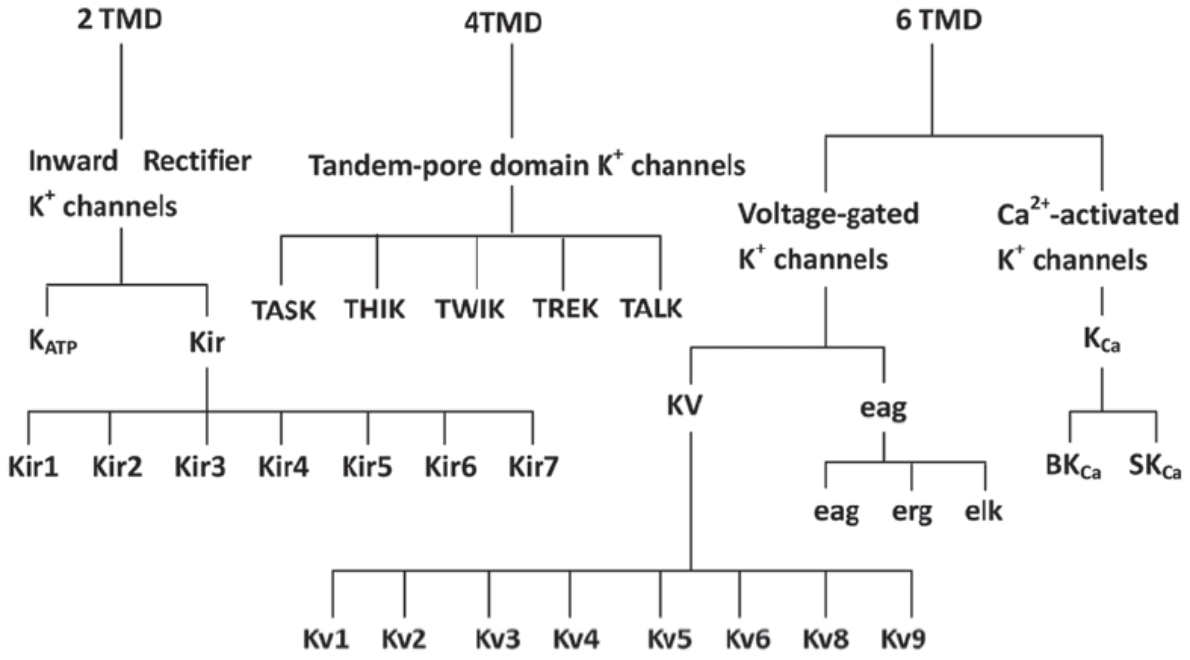

Figure 1.1. Classification of potassium channels. The three classes of potassium channels based on topological structures (the number of transmembrane domains). TMD, transmembrane domain; K_{ATP}, ATP-sensitive potassium channel; K_{ir}, inward rectifier potassium channel; TASK, tandem pore domain potassium channel; K_V, voltage gated potassium channel; EAG, ether-á-go-go channel; K_{Ca}, Ca^{2+} activated potassium channel; BK_{Ca}, large conductance K_{Ca} channel; SK_{Ca}, small conductance K_{Ca} channel.

(Zhong YS, Wang J, Liu WM, et al. (2013) Potassium ion channels in retinal ganglion cells (review). Mol Med Rep 8: 311-319)

6TMD channels: 6TMD channels are tetrameric channels, and each subunit has a conserved pore domain and six transmembrane domains. Compare to 2TMD and 4TMD channels, 6TMD channels are the largest class of potassium channels and can be divided into the voltage gated potassium channel group and Ca^{2+} activated potassium channel group. K_{Ca} channels are subdivided into BK channels, IK channels and SK channels respectively based on large (B), intermediate (I) and small (S) conductance. SK and IK channels can be activated by intracellular Ca^{2+} due to their association with a calcium binding protein called calmodulin. BK channel associate with auxiliary beta protein to form a functional channel. The major function of K_{Ca} channels is to regulate intracellular calcium (Millar *et al.*, 2007).

1.3 Two pore domain potassium channels

Two pore domain potassium channels (K_{2P}) also were called tandem domain potassium channel since each subunit contains two pore loops. K_{2P} channels have four transmembrane domains and look like inward rectifier channels joined together by a linker. Whereas inward rectifier channels assemble as tetramers, K_{2P} channels assemble as dimmers.

K_{2P} channels possess three special characteristics compared to other potassium channels.

1. Each subunit of K_{2P} channels contributes with two pore-forming domains to the ion pore after being assembled as a dimer. The other potassium channels subunits have only one pore-forming domain.

2. The mechanisms of K_{2P} channel regulation are multiple. Their activity can be regulated by pH, membrane stretch, temperature or intracellular signaling pathway(Goldstein *et al.*, 2005).

3. K_{2P} channels show a unique extracellular 'helical cap' structure between the M1 and M2 domain (Figure 1.2B). This structure could be a potential drug target to treat diseases related with K_{2P} channel dysfunction (Brohawn *et al.*, 2012; Miller and Long, 2012).

The K_{2P} family has 15 members that are subdivided into six subfamilies, which are TWIK, TREK, TASK, TALK, THIK and TWIK-related spinal cord K^+ channels (TRESK). In 1996, the first K_{2P} channel TWIK-1 was identified(Lesage *et al.*, 1996). In the next seven years (1996-2003) the other 14 K_{2P} members were identified (Enyedi and Czirják, 2010).

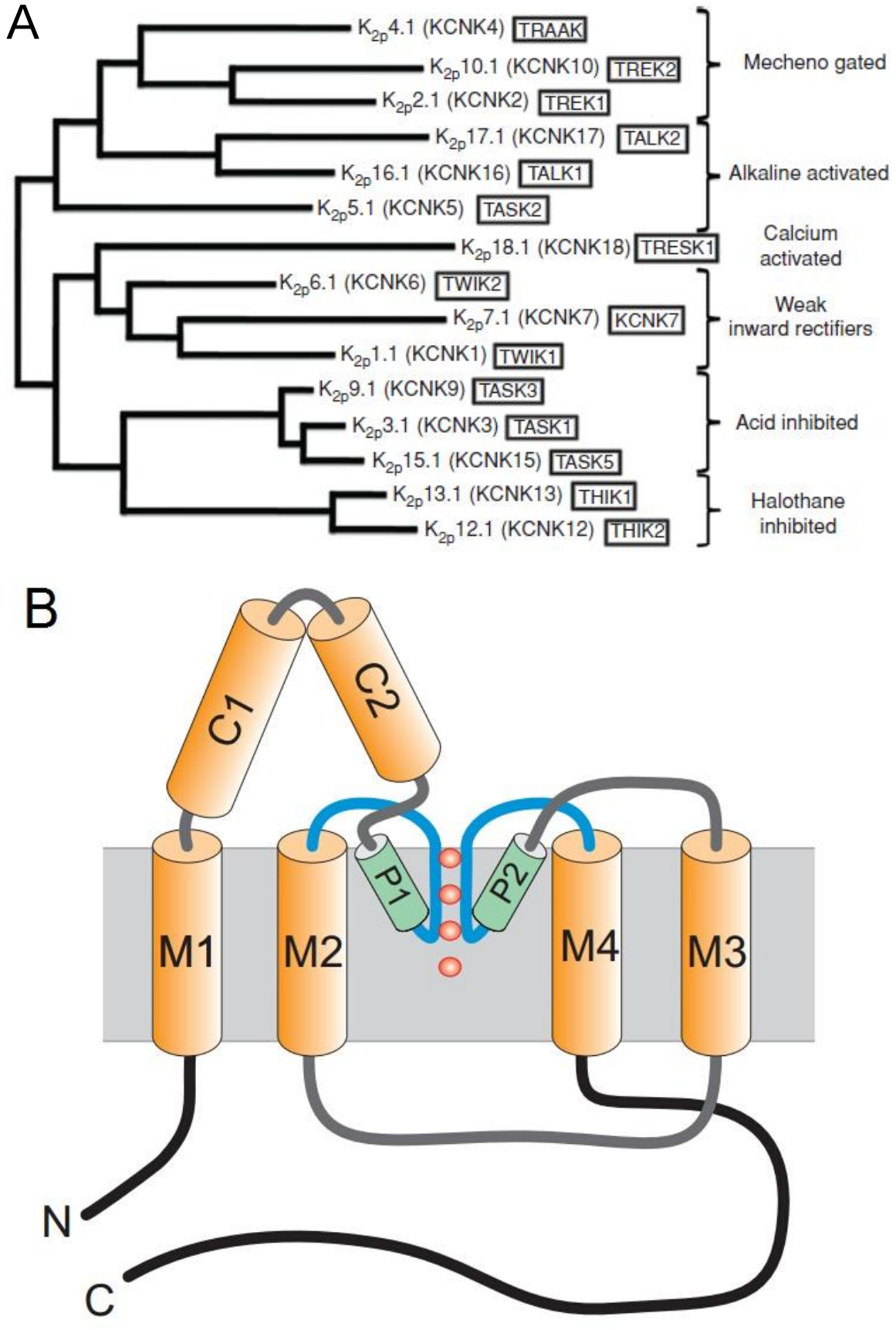
A
K2p4.1 (KCNK4) TRAAK
K2p10.1 (KCNK10) TREK2
K2p2.1 (KCNK2) TREK1
K2p17.1 (KCNK17) TALK2
K2p16.1 (KCNK16) TALK1
K2p5.1 (KCNK5) TASK2
K2p18.1 (KCNK18) TRESK1
K2p6.1 (KCNK6) TWIK2
K2p7.1 (KCNK7) KCNK7
K2p1.1 (KCNK1) TWIK1
K2p9.1 (KCNK9) TASK3
K2p3.1 (KCNK3) TASK1
K2p15.1 (KCNK15) TASK5
K2p13.1 (KCNK13) THIK1
K2p12.1 (KCNK12) THIK2
Mecheno gated
Alkaline activated
Calcium activated
Weak inward rectifiers
Acid inhibited
Halothane inhibited
B
C1
C2
P1
P2
M1
M2
M4
M3
N
C

Figure 1.2. Classification of two-pore-domain (2P) K^+ channel (K2P channel) and membrane topology. (A) Classification of K2P channel (from González, C., Baez-Nieto, D., Valencia, I., Oyarzún, I., Rojas, P., Naranjo, D., and Latorre, R. (2012). K^+ Channels: Function-Structural Overview. Comprehensive Physiology.) (B) Topological structure of K_{2P} channel. TRAAK, TWIK-related arachidonic acid activated K^+ channel; TREK, TWIK-related K^+ channel; TALK, TWIK-related alkaline pH-activated K^+ channel; TASK, TWIK-related acid-sensitive K^+ channel; TRESK1, TWIK-related spinal cord K^+ channel 1; TWIK, tandem of pore domains in a weak inward rectifying K^+ channel. THIK, tandem pore domain halothane-inhibited K^+ channel.

In the following section I will discuss some of the characteristics of those K_{2P} channels that are relevant for my thesis: The THIK subfamily, the TREK-1 subfamily, the TASK subfamily and the TWIK subfamily.

TWIK channel: The TWIK family has three members, TWIK-1 (KCNK1, $K_{2P}1.1$), TWIK-2 (KCNK6, $K_{2P}6.1$) and KCNK7 ($K_{2P}7.1$). TWIK channels originally were described as silent channels because no or only very weak inwardly rectifying currents could be measured in heterologous expression systems. TWIK-1 was first reported to induce a weak inward rectifying current in oocytes. TWIK-1 single channel conductance was about 34 ps at -80 mV in symmetrical 140 mM K^+ (Lesage *et al.*, 1996). Recently, it was shown that an endocytosis signal results in the weak currents (Feliciangeli *et al.*, 2010). On the other hand TWIK-1 possesses a hydrophobic barrier deep within the inner pore which may influence ion conduction. Pore mutation at Leu 146 in the middle of the M2 helix increased the currents of TWIK-1 mutant (Chatelain *et al.*, 2012; Aryal *et al.*, 2014). Human TWIK-2 also induces only a weak inwardly rectifying current in symmetrical K^+ in oocytes. However, Patel reported that rat TWIK-2 current was 15 times larger than human TWIK-2 current (Patel *et al.*, 2000). So the functional expression of TWIK-2 may be dependent on the expression system. KCNK 7(K_{2P} 7.1), cannot be heterologously functionally expressed in oocytes or COS-7 cells (Salinas *et al.*, 1999; Bockenhauer *et al.*, 2000). The KCNK7 channel has a Calcium-binding EF-hand motif in its C-terminus. This motif is unique in mammalian K_{2P} channels. Interestingly, the filter region of KCNK7 channels is GLE instead of the G(Y/F/L) G sequence which is strictly conserved in other K_{2P} channels.

TREK channels: The TREK family has three members, including TREK-1 (KCNK2; K_{2P}2.1), TREK-2 (KCNK10, K_{2P}10.1) and TRAAK (TWIK-related arachidonic acid activated K^+ channel, KCNK4, K_{2P}4.1). All the three members have a high variety of functionally active alternative splice variants. The sequence difference of those variant is at the N-terminus. Nevertheless, those splice variants have similar basic physiological characteristics and are regulated by the same processes(Patel *et al.*, 1998). In this work, the splice variant TREK-1c and TRAAK were used as control. TREK-1 is an outwardly rectifying potassium channel (Fink *et al.*, 1996) and has time-dependent gating. When TREK-1 was activated by depolarization, an instantaneous component was followed by a time-dependent component (Fink *et al.*, 1996; Maingret *et al.*, 2002; Renigunta *et al.*, 2015). The single channel conductances were 41 pS (low-conductance) or 132 pS (large conductance) at positive potentials (Xian Tao *et al.*, 2006). The two different conductances are attributable to two different isoforms of TREK-1 caused by alternative translation initiation (Thomas *et al.*, 2008; Renigunta *et al.*, 2015). TREK-2 also has different functional splice variants. TREK-2 channels expressed in mammalian cells can give rise to small (52 ps) and large (220 ps) conductance levels because of alternative translation initiation, resulting different isoforms with long and short N-terminus (Simkin *et al.*, 2008). The single channel conductances were 110 pS at -40 mV and 68 pS at +40 mV (Bang *et al.*, 2000; Lesage *et al.*, 2000). TRAAK is the last member of the TREK family which is restrictedly expressed in brain, retina and spinal cord. TRAAK can be activated by arachidonic acid and high intracellular pH values. For instance, if the pH increases from 7.3 to 8.8 the channel activity increased 14 times. Pressure and pH value change had a synergistic effect on channel activation (Kim *et al.*, 2001). The slope conductance was about 45.5 pS measured between 0 to 60 mV. The classical potassium blockers tetraethylammonium, 4-aminopyridine and Cs^+ do not affect the TRAAK channel, but it can be blocked by high concentration of Ba^{2+} (Fink *et al.*, 1998; Kim *et al.*, 2001).

TASK channels (TWIK-related acid-sensitive K^+ channel): The TASK family has three members, TASK-1, TASK-3 and TASK-5. TASK-2 and TASK-4 do not belong to this subfamily because of their alkaline range of pH sensitivity. TASK-1 and TASK-3 are highly sensitive to extracellular pH changes in a physiological range (Duprat *et al.*, 1997; Rajan *et al.*, 2000). TASK-1 and TASK-3 show the closest related structure

and share regulatory mechanisms. TASK-1 is sensitive to extracellular pH change and the inhibition is voltage independent, whereas intracellular pH change did not affect TASK-1 channel activity (Duprat *et al.*, 1997). In single channel recordings, TASK-1 channels expressed in mammalian cell showed a short-lasting opening (mean open time, 2 ms at -100 mV) and an unitary conductance of 16 ps. TASK-3 shows 50-60% sequence identity with TASK-1 in different species (Kim *et al.*, 2000; Rajan *et al.*, 2000). TASK-3 and TASK-1 can form functional heterodimers. The heterodimer displayed single channel kinetics nearly identical to TASK-3. In native tissues, endogenous TAKS-1/TASK-3 heterodimers were described (Kang *et al.*, 2004). TASK-3 is less sensitive to pH change compared to TASK-1, but for both channels a similar single amino acid residue, histidine 98, is critical for acid sensing. At single channel recordings, TASK-3 expressed in COS-7 cell showed a time-independent and non-inactivating current. The single channel conductance was 27 pS at -60 mV and 17 pS at +60 mV in symmetrical 140 mM K^+ concentrations (Kim *et al.*, 2000). TASK-5 is 51% amino acids sequence identity with TASK-1 and TASK-3. TASK-5 mRNA was expressed in many tissues including pancreas, liver, kidney, lung, ovary, and testis (Ashmole *et al.*, 2001). However, TASK-5 expressed in heterologous systems showed no measurable activity. TASK-5 cannot form heterodimers with TASK-1 (Ashmole *et al.*, 2001; Kim and Gnatenco, 2001). Probably the TASK-5 channel needs an auxiliary subunit to form a functional channel or it functions as an intracellular channel. It was reported that TASK-5 could be related to specific auditory neurons (Karschin *et al.*, 2001; Cui *et al.*, 2007).

THIK channels: The THIK family only has two members, THIK-1 (KCNK 13, $K_{2P}13.1$) and THIK-2 (KCNK 12, $K_{2P}12.1$). THIK channels were first reported by our group (Rajan *et al.*, 2001). THIK-1 expression was ubiquitous whereas THIK-2 expression was restricted, including lung, spleen and brain. THIK-2 channel is described to be a silent channel. In the next section we will introduce THIK channels in detail.

1.4 Tandem pore domain halothane inhibited K^+ channel

Tandem pore domain halothane inhibited K^+ channels (THIK channels) were firstly isolated from rat brain in our laboratory (Rajan *et al.*, 2001).

Heterologous expression of THIK-1 channel in *Xenopus* oocytes could produce outwardly rectifing currents in physiological external potassium concentrations and inwardly rectifing currents in symmetrical potassium concentrations. The current was not instantaneous but had an activation time constant of 0.97 ms at +60 mV and did not inactivate during a 500 ms voltage pluses. Extracellular acidification from pH 8 to pH 6 only weakly inhibited outward currents of THIK-1 whereas TASK-1 could be half-maximal blocked at pH 7.4. Intracellular pH also did not affect THIK-1 current. THIK-1 is not a temperature sensitive channel and is not influenced by lysophospholipids (Rajan *et al.*, 2001). THIK-1 channels can be activated by arachidonic acid (K_d 0.98 µM, Hill coefficient 1.97), similarly to the TRAAK channel. The currents are inhibited by halothane (K_d 2.8 mM, Hill coefficient 1.06), in contrast to other K_{2P} channels such as TREK-1, TREK-2 and TASK-1, which are activated by halothane (Patel *et al.*, 1999; Lesage *et al.*, 2000). The mechanisms of how halothane and arachidonic acid act on different K_{2P} channels are still unclear.

In single channel recordings, THIK-1 channel revealed a short and spiky channel opening and a weak inward rectification, like a background noise. The single channel conductance of THIK-1 was reported to be about 5 pS (Blin *et al.*, 2014; Kang *et al.*, 2014).

Heterologous expression of THIK-2 channels in *Xenopus* oocytes did not produce any measurable currents, and almost no expression of THIK-2 was found in cell membrane (Rajan *et al.*, 2001; Renigunta *et al.*, 2014). The same results were obtained by using human THIK-2 channel (Girard *et al.*, 2001). In this dissertation we will give some explanations, why currents related to THIK-2 channels could not be recorded previously. Recently Blin reported that THIK-1 and THIK-2 also can assemble heterodimers and form active channels (Blin *et al.*, 2014). However, it is not clear if this heterodimers are formed under physiological conditions.

The main physiological role of THIK-1 is not clear until now. In mouse cerebellar Purkinje neurons a THIK-1-like background current was described. GABAB and μ-opioid receptor agonists can potentiate THIK-1 currents (Bushell *et al.*, 2002). In nitric oxide synthase (NOS)-positive neurones of the glossopharyngeal nerve (GPN) a hypoxia-sensitive K^+ channel shared similar pharmacological properties with THIK-1 and regulates neuronal function (Campanucci *et al.*, 2003). Recently results also showed that in the retrotrapezoid nucleus (RTN) THIK-1 is expressed and a THIK-1-like, isoflurane-inhibited background potassium current could be measured, suggesting that THIK-1 channel inhibition may contribute to anesthetic-induced activation of retrotrapezoid nucleus neurons (Lazarenko *et al.*, 2010).

The physiological role of THIK-2 is still not clear. After bilateral cochlear ablation the expression of THIK-2 decreased significantly (Cui *et al.*, 2007). In situ hybridization data showed that THIK-2 was expressed in all parts of the inferior colliculus (central nucleus, dorsal cortex and lateral (external) cortex). (Karschin *et al.*, 2001; Holt *et al.*, 2006; Cui *et al.*, 2007).

Until now, the crystal structure of THIK channel is still unresolved. But by analyzing the published structures of TWIK-1 and TRAAK, we can assume that the structure of THIK-2 is similar. All K_{2P} channels studied so far have a helical cap (Figure 1.4).

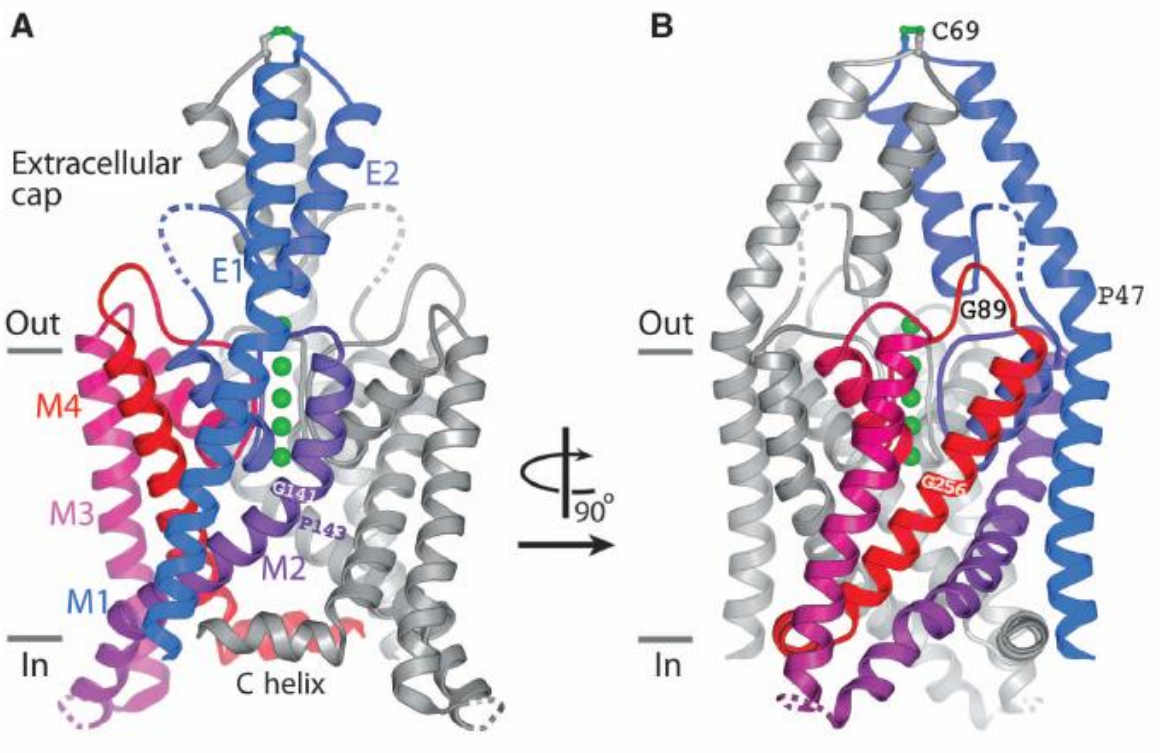

Figure 1.4. Tertiary structure of K_{2P} channel. (A-B) Tertiary structure of TWIK-1; (C) Tertiary structure of TRAAK (from Miller, A.N., and Long, S.B. (2012). Crystal Structure of the Human Two–Pore Domain Potassium Channel K2P1. Science *335*, 432-436.).

1.5 cAMP dependent pathway and IBMX

There are various intracellular pathways that transduce signals downstream from activated cell membrane receptors. The binding of ligands (first messengers) to the cell membrane receptors can lead to a short-lived increase or decrease of the concentration of intracellular signaling molecules named second messengers which are 3',5'-cyclic adenosine monophosphate (cAMP), 3',5'-cyclic guanosine monophosphate (cGMP), 1,2-diacylglycerol (DAG), inositol1,4,5-trisphosphate (IP3) and so on (Alberts *et al.*, 2007).

cAMP is synthesized from ATP by the enzyme adenylyl cyclase, and broken down to 5′ AMP by phosphodiesterases (PDEs). In this dissertation we used IBMX which is

an inhibitor of PDEs to increase cAMP level. Generally adenylyl cyclase can be activated by various stimuli, but ligand occupation of heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) coupled to Gs is the best-studied mechanism. G proteins are composed of α, β and γ subunits. When a specific ligand binds GPCR from extracellular, catalyzing the exchange of GDP for GTP on the alpha (α) subunit of G protein, the conformation of the G protein will be changed and the GTP-α subunit dissociates from the β-γ subunits. Then the α subunit interacts with and activates adenylyl cyclases. Activated adenylyl cyclases will synthesize cAMP from ATP. cAMP has at least three targets: PKA, the exchange protein activated by cAMP (Epac), and cyclic nucleotide-gated ion channels (CNGCs) (Murray, 2008).

PKA: Protein Kinase A is a family of enzymes whose activity is dependent on the cellular levels of cAMP. In activated state, PKA is a tetramer consisting of two regulatory (R) and two catalytic (C) subunits. Four cAMP bind to the two R subunits to activate PKA. cAMP binding changes the conformation of PKA, dissociating the two catalytic subunits from the 4 cAMP-2 R complex. The dissociated catalytic subunits then phosphorylate proteins by transferring ATP terminal phosphates to protein substrates at serine or threonine residues. Thisphosphorylation results in a change in activities of the substrates (Alberts et al, Molecular Biology of The Cell 5th, 2007, 908-912).

3-isobutyl-1-methylxanthine (IBMX) is a methylated xanthine derivative. Generally IBMX is used as competitive nonselective inhibitor of phosphodiesterase, resulting in an increase of cyclic adenosine monophosphate (cAMP) levels pathway (Essayan, 2001). For example, when TREK-1 was discovered in 1996, Fink et al. used IBMX (1 mM) and forskolin (10 μM) to study the cAMP pathway of the channel (Fink *et al.*, 1996). To study the cAMP pathway people used usually IBMX and forskolin together to completely activate pathway because forskolin can active adenylyl cyclase and meanwhile IBMX can inhibit phosphodiesterase.

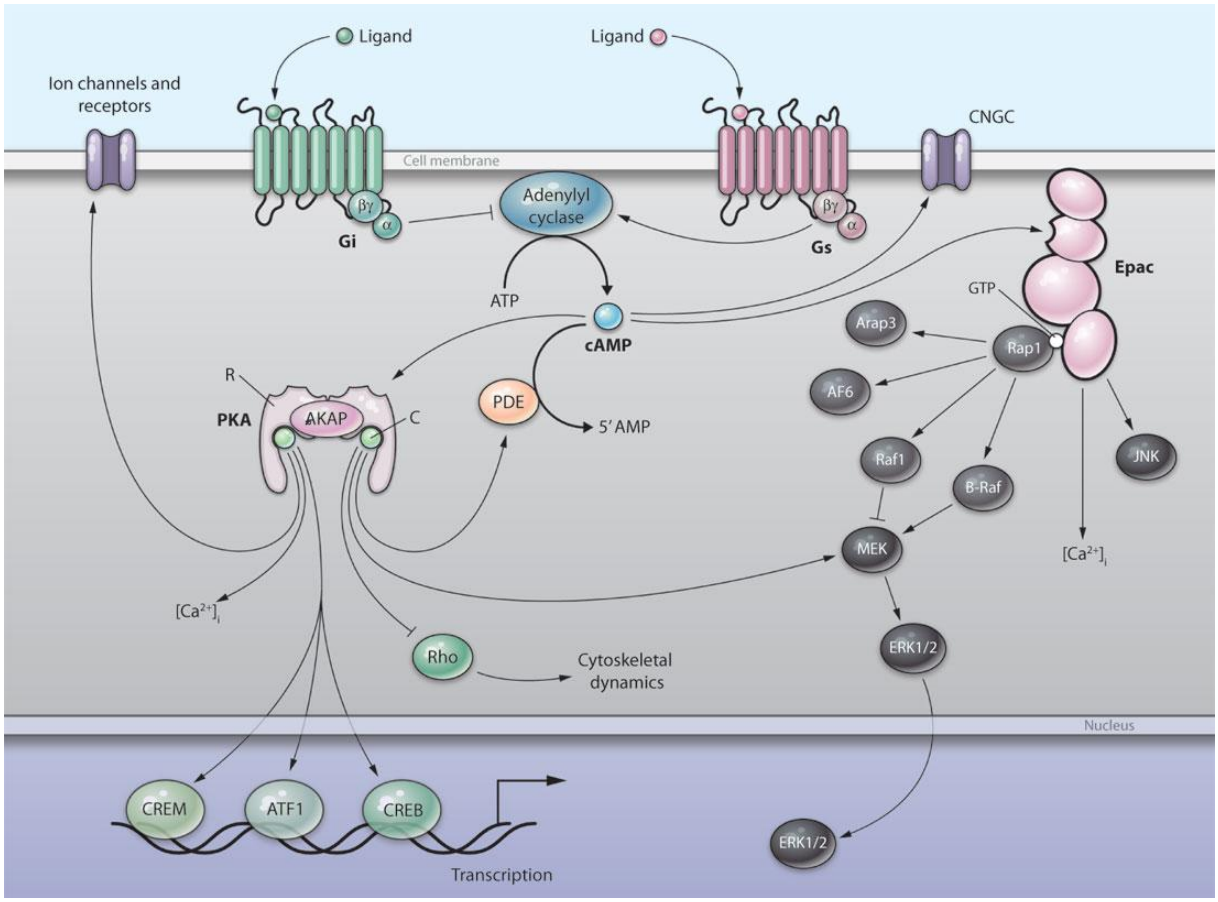

Figure 1.5 Summary of the cAMP signaling cascade. AKAP, A-kinase anchoring protein; ATF1, activating transcription factor 1; C, protein kinase A catalytic subunit; cAMP, cyclic adenosine monophosphate; CNGC, cyclic nucleotide–gated ion channel; CREB, cAMP response element–binding protein; CREM, cAMP response element modulator; ERK1/2, extracellular signal–related kinase 1/2; JNK, Jun N-terminal kinase; PDE, phosphodiesterases; PKA, protein kinase A; R, protein kinase A regulatory subunit (from Murray, A.J. (2008). Pharmacological PKA inhibition: all may not be what it seems. Science signaling *1*, re4.).

1.6 Aims of this dissertation

In this dissertation we will prove that IBMX can bind to K_{2P} channels and block their activity directly and not by affecting signaling pathways - cAMP pathway.

Generally IBMX is used as inhibitor of an enzyme phosphodiesterase, resulting in an increase of cAMP levels (Essayan, 2001), further to activate cAMP pathway including PKA pathway and Epac pathway. For example, IBMX was used in researches of TREK-1 channel (Fink *et al.*, 1996), T-type Ca^{2+} channels (Gautam *et al.*, 2007), ATP-sensitive K^+ channel (K_{ATP}) channel (Morales *et al.*, 2004; Herbst *et al.*, 2011) and so on. However it is not described to be a direct blocker of K_{2P} channels. In this dissertation we will prove this property of IBMX in THIK channels.

THIK-2 family has two members, THIK-1 and THIK-2. However THIK-2 is described as a silent channel. Before testing the effect of IBMX on THIK-2 channel, we tried to 'break the silence' of the channel. I have studied the effect of various mutations of THIK-2 channels expressed a mammalian cell line on the amplitude of THIK-1 currents. This part of work has been published (Renigunta *et al.*, 2014).

The objectives were met in collaboration with Dr. Vijay Renigunta. All the constructs of the channels were made by him. The measurements of surface expression were also supervised by Dr Renigunta. Those data are included in the result section of this dissertation to provide a complete overview of the research.

2 Materials and Methods

2.1 Materials

Name	Compay
35 mm glass-bottom dishes	ibidi, Germany
cAMP	Sigma-Aldrich
$CaCl_2$	Sigma-Aldrich
DNA ploymerase and restriction endonuclease	Fermentas
EGTA	Sigma-Aldrich
F-12 medium	PAA laboratories Gmbh
FBS	PAA laboratories Gmbh
Forskolin	Sigma-Aldrich
H89 dihydrochloride	TOCRIS
HEPES	Sigma-Aldrich
HRP-secondary antibody	Jackson ImmunoResearch, UK
IBMX	Sigma-Aldrich
jetPRIME reagent	Polyplus transfection, France
K_2ATP	Sigma-Aldrich
KCl	MERCK, Germany
K-Glutamate	Sigma-Aldrich
KOH	MERCK, Germany
$MgCl_2$	MERCK, Germany
Na_2GTP	Sigma-Aldrich
NaCl	Sigma-Aldrich

NaH_2PO_4	SERVA, Germany
NaOH	Sigma-Aldrich
penicillin/streptomycin	PAA laboratories Gmbh
Pipette 1.05×1.50×80 mm	Science Products GmbH
QuickChange site-directed mutagenesis	Agilent technologies
Quinidine hydrochloride monohydrate	Sigma-Aldrich
high-glucose DMEM	Life Technologies, UK
monoclonal anti-CD74 antibody	Dianova, Germany
TPA	Sigma-Aldrich

2.2 Solutions

ND96 culture solution

96 mM	NaCl
2 mM	KCl
1 mM	$MgCl_2$
1.8 mM	$CaCl_2$
5 mM	HEPES
2.5 mM	sodium pyruvate
100 µg/ml	gentamycin

pH 7.4-7.5 adjusted with 1 mM NaOH

ND96 recording solution

96 mM	NaCl
2 mM	KCl
1 mM	$MgCl_2$

1.8 mM	$CaCl_2$
5 mM	HEPES
pH 7.4-7.5 adjusted with 1 mM NaOH	

Pipette solution

60 mM	KCl
65 mM	K-Glutamate
5 mM	EGTA
3.5 mM	$MgCl_2$
3 mM	K_2ATP
0.2 mM	Na_2GTP
2 mM	$CaCl_2$
5 mM	HEPES
pH 7.2 adjusted with 1 mM KCl	

5 mM K^+ Bath solution for whole cell recording

5 mM	KCl
135 mM	NaCl
10 mM	Glucose
1 mM	MgCl
0.33 mM	NaH_2PO_4
1 mM	CaCl
2 mM	sodium pyruvate
10 mM	HEPES
pH 7.4 adjusted with 1 mM NaOH	

140 mM K^+ Bath solution for whole cell recording

140 mM	KCl

10 mM	Glucose
1 mM	MgCl
0.33 mM	NaH_2PO_4
0.2 mM	CaCl
2 mM	sodium pyruvate
10 mM	HEPES

pH 7.4 adjusted with 1 mM NaOH

2.3 Molecular cloning and mutagenesis

The cDNA encoding rat THIK-1 (NM_022293), rat THIK-2 (NM_022292) and human TREK-1c (AY_552980), hTRAAK (NM_033310) were individually subcloned into the vector pCDNA 3.1 (+) or pCDNA 3.1 (-) for expression in Chinese hamster overay (CHO) cell or subcloned into pSGEM oocytes expression vector for synthesizing cRNA used in voltage clamp system. For surface expression of CD74 reporter protein series, the N-terminus of THIK-2 was fused to the reporter protein CD74. Point mutants of rat THIK-1, THIK-2 or human TREK-1c was introduced by QuickChange site-directed mutagenesis (Agilent technologies). Mutations were confirmed by DNA sequencing of the entire gene (Seqlab Company). All the constructs were kindly provided by Dr. Vijay. Reningunta.

2.4 Voltage-clamp measurements in *Xenopus laevis* oocytes

Oocytes were surgically removed from *Xenopus laevis*. Anaesthesia and operationwere carried out in accordance with the principles of German legislation with approval of the animal welfare officer of the Medical Faculty of Marburg University under the governance of the Regierungspräsidium Giessen (the regional veterinary heath authority). For *Xenopus* oocytes voltage clamp system complementary RNA (cRNA) was synthesized by transcription in vitro from Xho-1 linearised (THIK-1 and mutants) plasmid with T7 polymerase (mMessage mMachine T7 Kit, Ambion). The concentration of cRNA was measured by NanoDrop 2000 and diluted into the concentration of 120 ng/µl with RNase-free water for injection. For

THIK-1 and mutants, each Dumont stage VI oocytes was injected with 6 ng cRNA, TRAAK with 2.5 ng/oocyte. Oocytes were incubated at 19 °C for 48 hous in ND96 culture solution. Currents were measured at 0 mV with or without IBMX in ND96 recording solution. The two electrodes were filled with 3 M KCl. Holding potential is -80 mV, sampling rate is 120 Hz.

2.5 Cell culture

CHO and COS-7 cells were grown in 35 mm glass-bottom dishes. CHO cells were cultured in Ham´s F-12 medium with L-Glutamine, 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. COS-7 cells were cultured in high-glucose DMEM with L-Glutamine, 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. Before 100% confluence the cells should be passaged and the medium was changed every 48 hours. The transient transfections were performed with jetPRIME reagent following the manufacturer's protocol. 2 µg DNA and 4 µl jetPRIME reagent per dish were used for transfection. Before 40^{th} generation of the CHO cell we should use new one.

2.6 Patch clamp measurements

THIK-1, THIK-2, TREK-1c and their mutants were measured with whole cell voltage clamp. Pipettes were pulled from borosilicate glass capillaries. The currents were measured at room temperature. The resistance of pipette was about 3-6 MΩ. Before gigaseal a positive pressure was used. Gigaseal of the patch should be bigger than 1 GΩ by applying negative pressure. Steady-state current-voltage relations were obtained with slow voltage ramps (40 mV s-1) between -120 and +40 mV. The liquid junction potential between the patch electrode and the bath solution (~ -8 mV) was not compensated.

2.7 Surface expression of CD74 reporter protein

CD74 constructs including MHRRRSR, MHSSSSS, N-terminal of THIK-2 (AAA) and N-terminal mutant of THIK-2 (RRR) were transfected into COS-7 cell respectively using jetPRIME reagent. After 24 h, cells were fixed with 4% PFA in PBS for 10 min at room temperature (RT) and then washed 3 times in PBS. Before application of a monoclonal anti-CD74 primary in 500 µl staining buffer (PBS + 1% goat serum) for 1 hour at RT, the cells were blocked with 10% goat serum in PBS for 30 min at RT. After washing out the primary antibody with SB secondary antibody was applied for 30 min at RT. Surface expression was measured by luminometry using a luminogenic substrate of HRP (SuperSignal; Pierce Biotechnology, Rockford, IL, USA) and maximum value was used.

2.8 Statistics

Data were analyzed and Figured with sigma plot software. Reported data are means±standard error of the mean (SEM). Student T-TEST and F-TEST were used to determine the statistical significance. In Figures statistically significant differences to control values are marked by asterisks (*, $p<0.05$; **, $p<0.01$; *** $p<0.001$).

3 Results

3.1 IBMX blocks THIK-1 from the extracellular side

3.1.1 **IBMX blocks THIK-1 channel currents**

To study the cAMP pathway in THIK-1 channel, we used IBMX as a competitive nonselective phosphodiesterase inhibitor that will raise intracellular cAMP level after application, thereby active PKA pathway (Essayan, 2001). In our experiments, we found that 1 mM IBMX could quickly inhibit THIK-1 channel inward and outward currents in CHO cells (Figure 3.1.1A) or oocytes (Figure 3.1.1C).Those data indicated that the cAMP pathway may be involved in the regulation of THIK-1 currents just like in TREK-1 (Fink *et al.*, 1996; Gonzalez *et al.*, 2012).

To analyze the kinetic of the inhibition of THIK-1 by IBMX we compared it to the change of the potassium concentration in the bath solution from 5 to 24 mM K^+ by recording inward currents at -80 mV (Figure 3.1.1B, black). Interestingly, the effect of 1 mM IBMX on the THIK-1 outward currents at 0 mV is equally fast compared to the change of the potassium concentration. If IBMX would act on THIK-1 via the elevation of the intracellular cAMP level, such a fast response would be unlikely. This observation opens the question whether IBMX could inhibit THIK-1 directly. However the reversibility of the IBMX effect was significantly slower than the solution change.

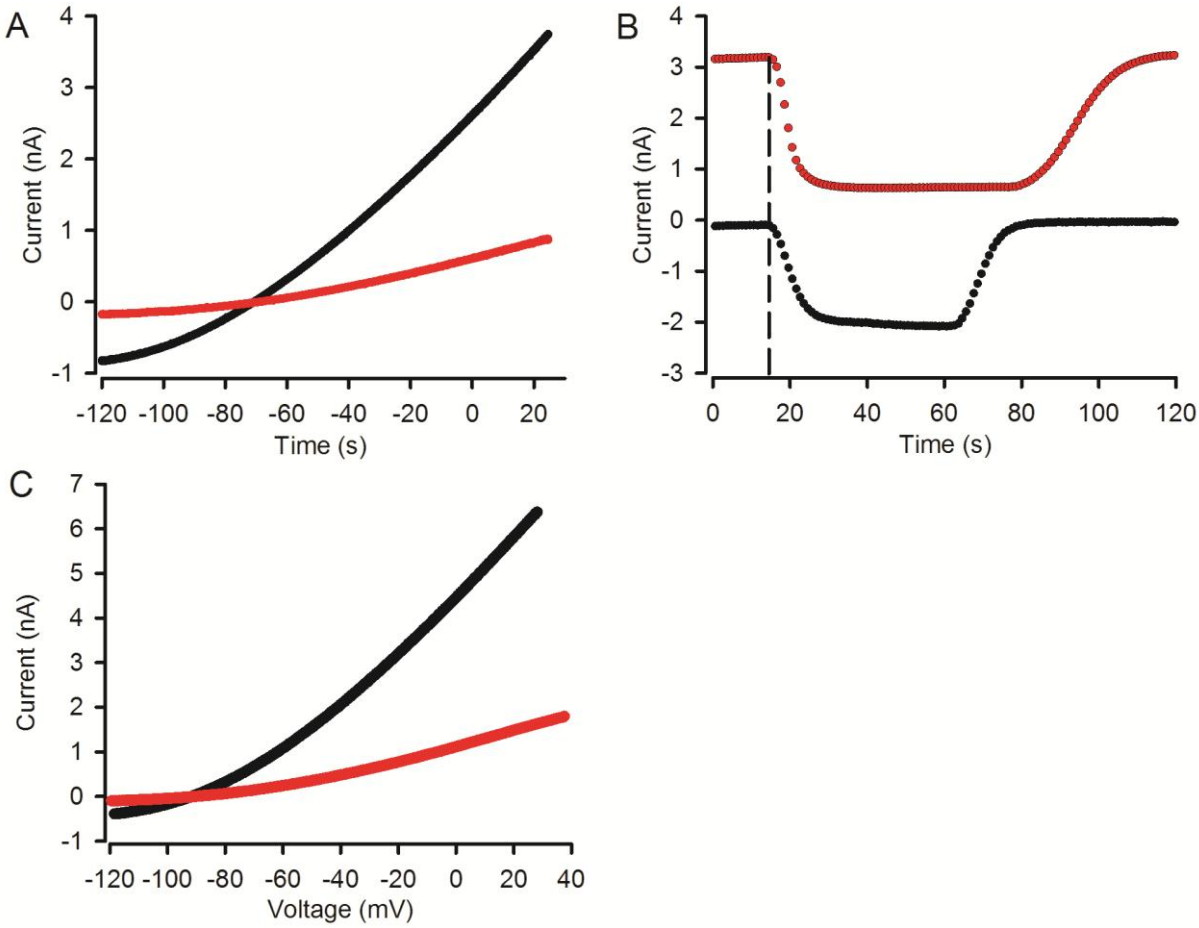

Figure 3.1.1. IBMX blocked THIK-1 channels expressed in CHO cell or oocytes. (A) current-voltage relationship (IV curve) of THIK-1 expressed in CHO cell with or without application of 1 mM IBMX. Red: 1 mM IBMX. Black: control. (B) Time course of the effect of IBMX outward current (red, holding potential at 0 mV) and of extracellular potassium concentration change from 5 mM to 24 mM (black, holding potential at -80 mV).(C) current- voltage relationship (IV curve) of THIK-1 expressed in oocytes with or without application of 1 mM IBMX. Red: 1 mM IBMX. Black: control.

3.1.2 Effects of H89 and forskolin on THIK-1 channel

To analyze more in detail the role of cAMP induced pathways in regulation THIK-1, we used two additional chemicals that are commonly used to interfere with such pathway: H89 and forskolin.

H89 is a PKA inhibitor. It blocks PKA signaling pathway though competitive binding to the ATP binding site on the PKA catalytic subunits (Murray, 2008). In our experiments, we applied 5 µM H89 to inhibit the PKA pathway. Interestingly, the currents of THIK-1 expressed in CHO cells were not affected (Figure 3.1.2A).

Furthermore, after pre-incubating the cells with 5µM H89 for at least 10 min, 200 µM IBMX could still inhibit THIK-1 in presence of H89 (Figure 3.1.2B). It seems that the effect of IBMX on THIK-1 is independent of the PKA pathway as the inhibition was not significantly changed in the presence of H89 (relative current of IBMX alone was 0.48 ±0.03, relative current of H89 and IBMX together was 0.499 ±0.04, Figure 3.1.2C).

Forskolin is a diterpene and widely used to increase cAMP level to study PKA pathway. Forskolin is a PKA pathway activator as it activates adenylyl cyclase, resulting in an increase of intracellular cAMP level. IBMX and forskolin are often used in combination to fully activate the PKA pathway. Forskolin actives adenylyl cyclase and IBMX inhibits phosphodiesterase, both processes increase the intracellular cAMP levels and subsequently activates PKA pathway cooperatively (Kang *et al.*, 2007; Dixon *et al.*, 2011; Cuppoletti *et al.*, 2014). In this study human TREK-1c was used as a positive control for active PKA as its currents are blocked upon phosphorylation at serine 344 by PKA. The TREK-1 S344A mutant cannot be phosphorylated and is not affected by PKA (Fink *et al.*, 1996; Maingret *et al.*, 2000).

In our experiments 20 µM forskolin had no effect on THIK-1 currents expressed in CHO cells (Figure 3.1.2D). In contrast, TREK-1c expressed also in CHO cells was used as positive control on PKA pathway (Rinné *et al.*, 2014). There was nearly completed and reversible inhibition by 20 µM forskolin (Figure 3.1.2E-F), confirming published data (Terrenoire *et al.*, 2001; Rinné *et al.*, 2014).

Aa a conclusion, the PKA activator forskolin and the PKA inhibitor H89 both do not alter the effect of IBMX on THIK-1 channel, indicating that the IBMX effect is not mediated through PKA.

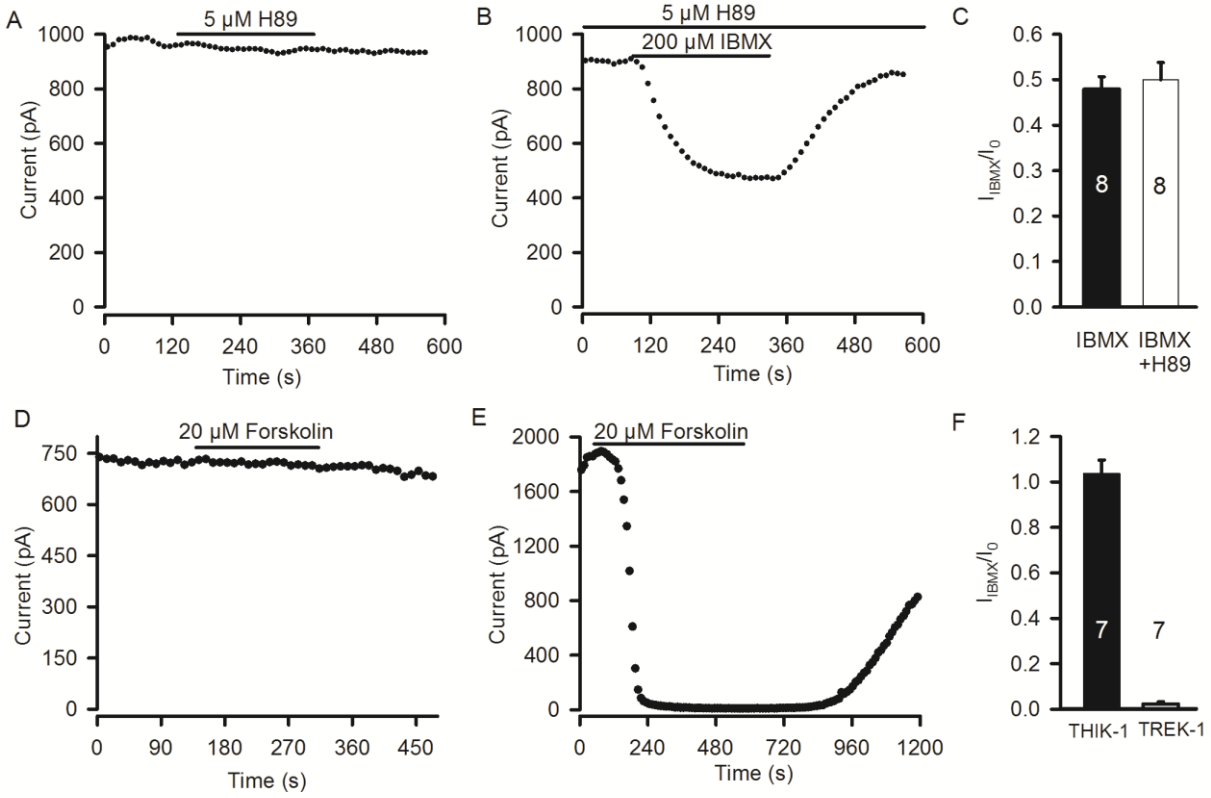

Figure 3.1.2. The effects of PKA inhibitor H89 and PKA activator forskolin on THIK-1 channels expressed in CHO cells at holding potential of 0 mV. (A) Effect of application of 5 μM H89 on THIK-1 channels with whole-cell patch recording. (B) The effect of 200 μM IBMX on THIK-1 channels in presence of H89 after pre-incubation for 10 min. (C) Statistics of the inhibition of IBMX with or without pre-incubating H89. (D) Application of 20 μM forskolin on THIK-1 channels by whole cell recording. (E) Application of 20 μM forskolin on TREK-1 channels by whole cell recording. (F) Statistics of the effects of 20 μM forskolin on THIK-1 or TREK-1 currents. I_{IBMX}, the currents of THIK-1 in presence of IBMX. I_0, the currents of THIK-1 before superfusion of IBMX. The number of the measured cell is given in black or white; the error bars indicate SEM

3.1.3 Effect of cAMP on THIK-1 channel

cAMP is an important second messenger involved in various biological processes, in particular the PKA and the Epac (exchange protein activated by cAMP) pathway. We observed, that only IBMX but not forskolin or H89 affect the currents of THIK-1, in contrast to TREK-1. To further test, if cAMP is the mediator of the IBMX effect, we

applied cAMP directly into the cytosol of CHO cells transfected with THIK-1 channels by including 100 µM cAMP into the patch pipette. The currents were measured at a holding potential of 0 mV and after rupture of the patch membrane the outward current was measured.

Immediately after rupturing the patch membrane THIK-1 outward currents could be measured and remained constant (fig. 3.1.3A). In contrast, TREK-1c currents rapidly decayed and nearly disappeared after 15 seconds (0.1 ±0.02), indicating a diffusion of cAMP into the cell which in turn induce the inhibition of the TREK-1c channel (Figure 3.1.3C). As a control, the TREK-1c S344A mutant in which the PKA phosphorylation site at position 344 (serine) was mutated to alanine (TREK-1c^{S344A}), was not affected by cAMP (fig. 3.1.3D). These data show that the PKA pathway in CHO cells can be activated within less than 15 seconds by rupturing the patch membrane with a subsequent diffusion of cAMP into the cell.

All data show that THIK-1 currents are inhibited by IBMX but are not affected by forskolin, H89 and cAMP, indicating that the IBMX effect is not mediated by the PKA or the Epac pathway.

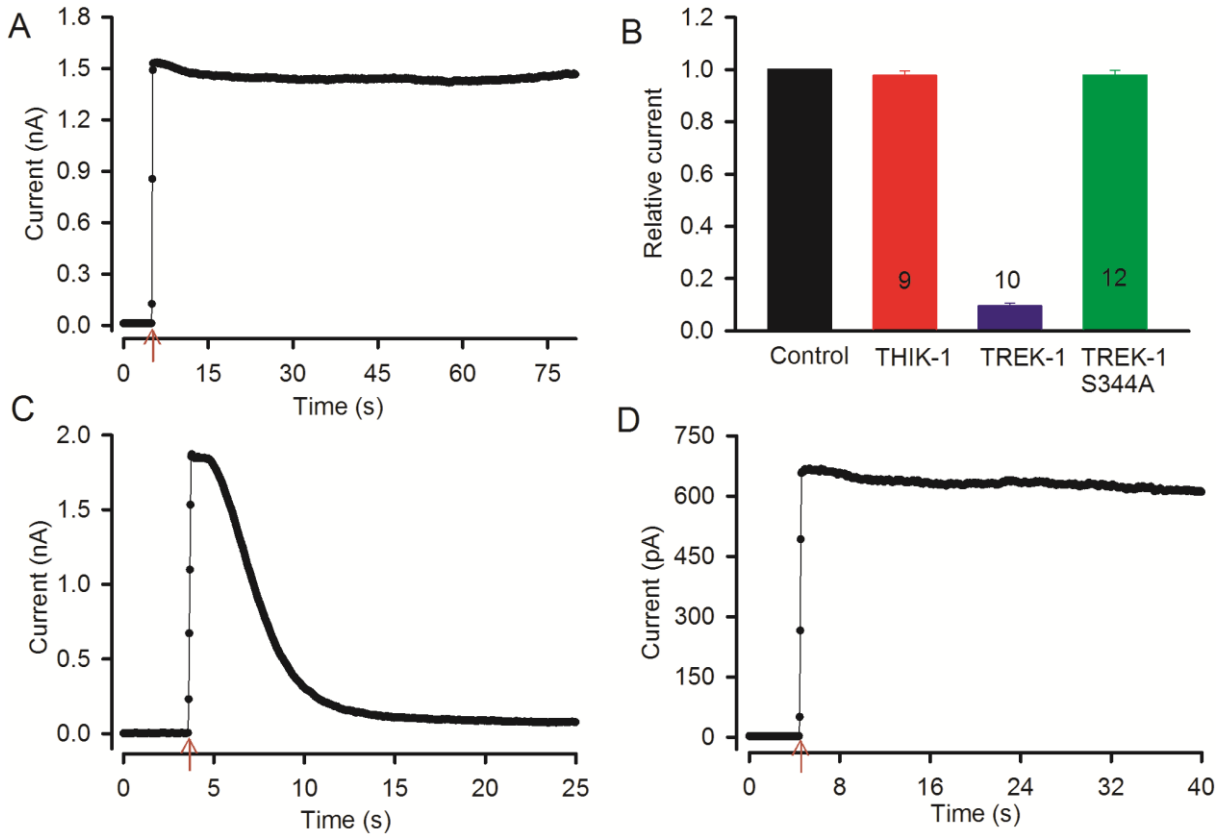

Figure 3.1.3. The effects of 100 µM intracellular cAMP, applied via patch the pipette, on THIK-1, TREK-1c or TREK-1c S344A expressed in CHO cells. (A) The effect of 100 µM intracellular cAMP on THIK-1 channel at holding potential of 0 mV. A cell-attached patch was formed and then ruptured by negative pressure at the red arrow. (B) The relative current measured 1 min after rupturing the patch membrane in CHO cells compared to the current measured immediately after rupturing the patch membrane at a holding potential of 0 mV. (C) The effect of 100 µM intracellular cAMP on TREK-1c channel at a holding potential of 0 mV. (D) The effect of 100 µM intracellular cAMP on TREK-1c S344A mutant channel at a holding potential of 0 mV. The number of the measured cell is given in black; the error bars indicate SEM.

3.1.4 Block of THIK-1 channels by IBMX

To analyze the effect of IIBMX on THIK-1 in more detail, we next tested whether IBMX inhibits the channel when applied from the intracellular side (by introducing 1 mM IBMX into the patch pipette). Interestingly, IBMX had no effect: the currents at a holding potential of 0 mV stayed constant after rupturing the cell membrane. But when the extracellular medium of the same cell is superfused with a 1 mM IBMX bathsolution, the THIK-1 currents decrease (Figure.3.1.4A), indicating that IBMX only can inhibit THIK 1 if it is applied from the extracellular side.

It has been described that tetrapentylammonium (TPA) can directly block potassium channels from intracellular side (Sanchez and Blatz, 1995; Oliver *et al.*, 1998; Piechotta *et al.*, 2011). Consequently, we used TPA as a positive control to inhibit THIK-1 and TREK-1 channels using our method. We found that both channels could be strongly inhibited with 20 µM intracellular TPA applied via the patch pipette (Figure 3.1.4D, E).

To further analyze the IBMX effect on the THIK channel we tested different concentrations of IBMX to calculate the IC_{50} value and the Hill coefficient. We found that the IC_{50} of IBMX on THIK-1 expressed in CHO cells was about 152 µM, and the Hill coefficient was about 1 (Figure 3.1.4F). However, in oocytes the sensitivity of THIK-1 to IBMX was lower than in CHO cell. For instance, the relative block of 500

μM IBMX in oocytes was about 60% compared to about 75% in CHO cell (Figure 3.1.5F, G).

TRAAK channel, another K_{2P} channel, is expressed as two isoforms named TRAAKa and TRAAKb (Ozaita and Vega-Saenz de Miera, 2002). Because TRAAK is not regulated by PKA and PKC kinases (Fink *et al.*, 1998), we used the two TRAAK isoforms as control to test the specificity of IBMX on THIK-1. It was found that the block of IBMX to THIK-1 is highly specific: IBMX had no effect on TRAAK channels expressed in Xenopus oocytes (Figure 3.1.4H).

In conclusion, IBMX blocks THIK-1 only and specifically from the extracellular side, strongly indicating a direct effect of IBMX on the channel rather than by the induction of any signaling pathway such as the PKA pathway.

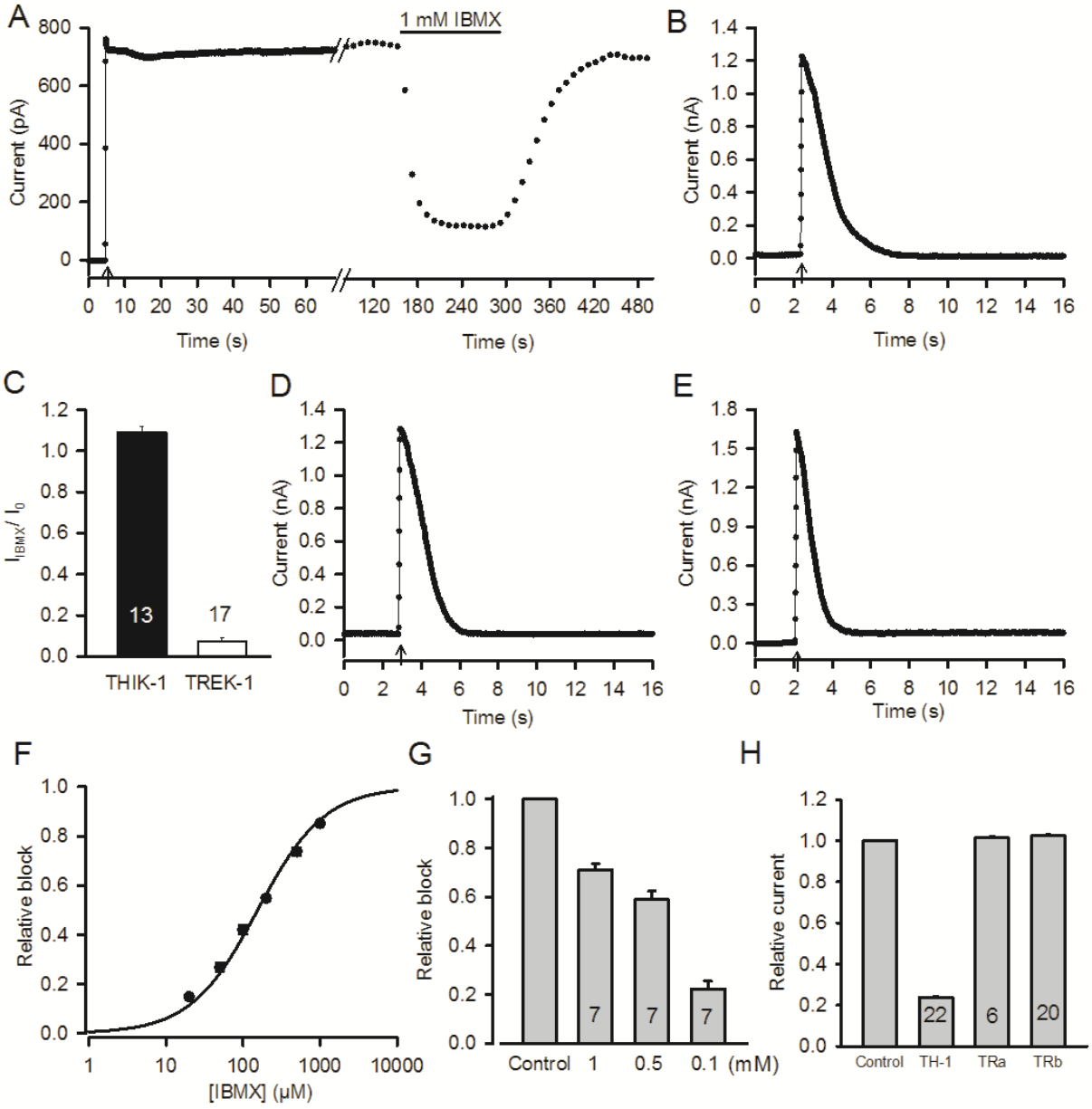

Figure 3.1.4. IBMX specifically blocks THIK-1 currents expressed in CHO cell from extracellular side. (A) The effect of application of 1 mM intracellular IBMX via the patch pipette (left) and or via the bath solution (right) in a CHO cell transfected with THIK-1. Curve shows a continuous recording. (B) The effect of application of 1 mM intracellular IBMX via patch pipette in a CHO cell transfected with TREK-1c. (C) Statistics of the effects of 1 mM intracellular IBMX on THIK-1 or TREK-1 currents. I_{IBMX}, the currents in presence of IBMX. I_0, the currents before superfusion of IBMX. The number of the measured cell is given in black or white; the error bars indicate SEM. (D) The effect of application of 20 μM intracellular TPA via patch pipette in a CHO cell transfected with THIK-1. (E) The effect of application of 20 μM intracellular TPA via patch pipette in a CHO cell transfected with TREK-1c. (F) steady-state dose response curve of THIK-1 channels expressed in CHO cells by IBMX. (G) Relative

block of different concentration IBMX in THIK-1 channels expressed in oocytes (6 ng cRNA /oocyte). (H) The effects of 1 mM IBMX on hTRAAKa and hTRAAKb expressed in oocytes (2.5ng cRNA/oocyte in TRAAK and 6 ng cRNA/oocyte in THIK-1). The number of the measured cells is given in black or white.

3.1.5 Identification of the IBMX binding site

IBMX blocks THIK-1 directly and specifically. To identify the IBMX binding site we mutated all the amino acids of the helical cap in THIK-1 one by one. Subsequently, the effects of IBMX weres measured in oocytes and the results were compared between the mutants of THIK-1 and its wild type. Alanine residues were mutated to serine, and the other residues were mutated to alanine. THIK-1 and its mutants were treated with 500 µM IBMX and the ratios of the currents were measured in the presence of 500 µM IBMX (I_{IBMX}) or in the absence of IBMX (I_0). Most of the mutants showed a residual current (I_{IBMX}/I_0) of about 0.4 after application of 500 µM IBMX, similar to THIK-1 wild type, indicating that those amino acids are not potential IBMX binding sites. However, the mutant R92A showed a significantly reduced sensitivity to IBMX compared to the wild type. Additionally, the mutants K50A and G85A showed a slightly decreased sensitivity to IBMX, although to a lower extent than R92A (Figure 3.1.5A).

Alignment of the helical cap regions of the K_{2P} channels that have been crystallized so far (TWIK-1, TRAAK and TREK-2) together with THIK-1 and its homologous THIK-2 shows that the cap helices 1 and 2 are highly conserved in these channels. In contrast, the linker region (C2-P1 linker, indicated by a red line) between cap helix 2 and the pore helix is more variable. Part of the linker region of K_{2P} channels was found to be disordered. The amino acids missing in the crystal structure are highlighted in yellow (Figure 3.1.5). Interestingly, the arginine residue at position 92, which showed the lowest sensitivity to IBMX in our mutants scan, is at the end of the unstructured region, three residues proximal to the pore helix.

To further study the character of the C2-P1 linker we constructed a THIK-1/TREK-1 chimera in which the linker region of THIK-1 was replaced by TREK-1 and analyzed

the inhibitory effect of IBMX. We found that the chimera showed significantly less sensitivity to different concentrations of IBMX (Figure 3.1.5), supporting the idea that amino acids of the linker regions are responsible for the binding of IBMX to THIK-1.

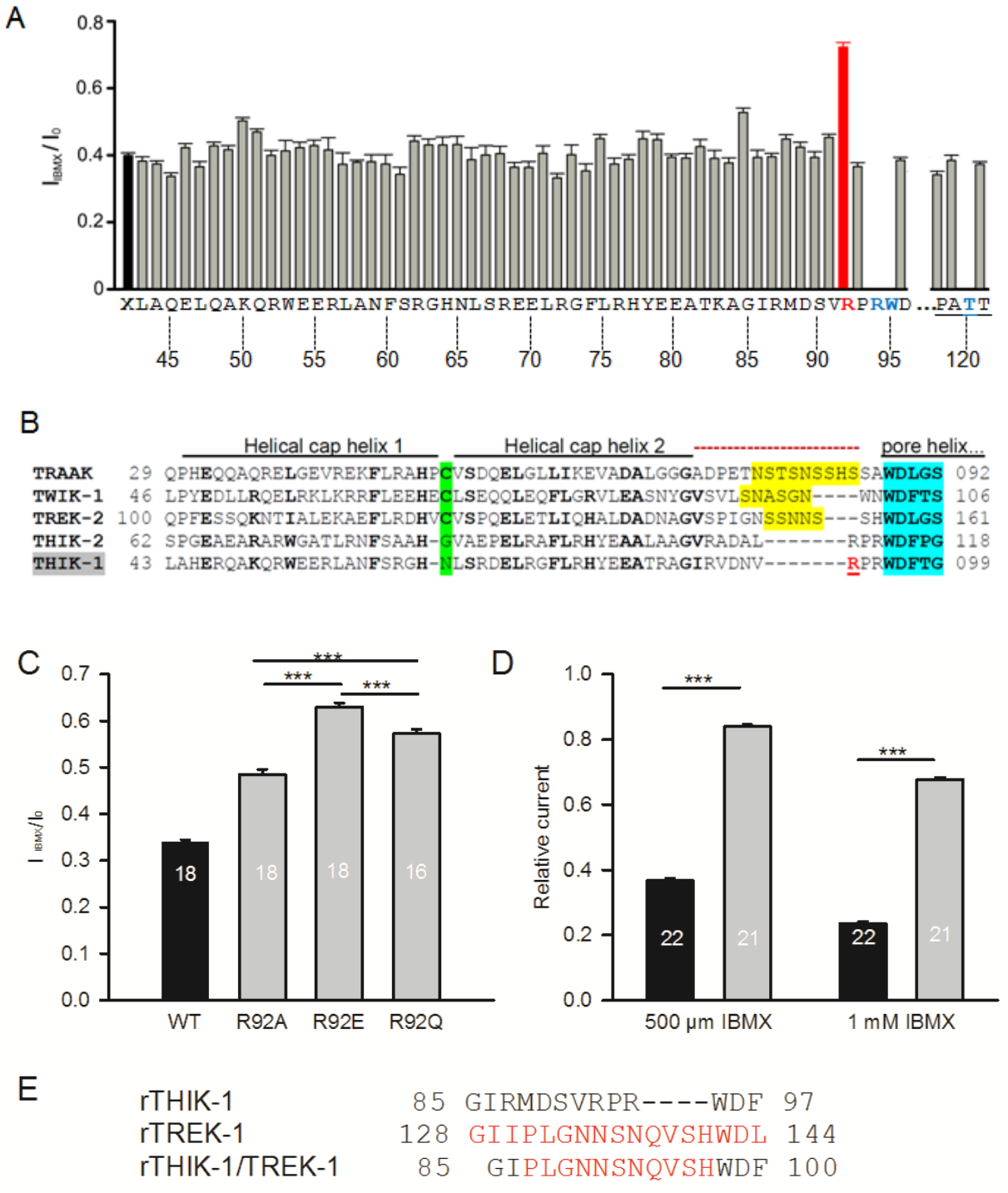

Figure 3.1.5. The effects of IBMX on the mutants of the helical cap of THIK-1. (A) The relative current measured in wt THIK-1 and mutants in the presence of 500 µM IBMX, defined as the ratio between the outward current measured at 0 mV in the presence of IBMX (I_{IBMX}) and the current measured before application of IBMX (I_0). The residues shown on the abscissa represent the amino acids of the extracellular cap. Alanine residues were mutated to serine, all other residues were mutated to alanine. (B) Alignment of the three K_{2P}-channels with known crystal structure togather with THIK-2 and THIK-1 (grey background). The residues cut out in the crystal structure are marked with yellow background; the unstructured region of the C2-P1 linker is indicated by a dotted red line; conserved residues are shown in bold; residue R92 is marked in red. (C) The effects of 500 µM IBMX on THIK-1 mutants at position 92. Currents were measured at 0 mV in oocytes. I_0, currents before application of 500 µM IBMX in oocytes. I_{IBMX}, currents of application of IBMX. (D) The effects of different concentrations of IBMX on THIK-1/TREK-1 (grey) and THIK-1 wild type bBlack). (E) The sequence of THIK-1/TREK-1 chimera. The C2-P1 linker of THIK-1 is replaced by TREK-1 (red characters). The number of cells measured is given in white; the error bars indicate SEM.

Arginine 92 of THIK-1 is a basic amino acid with positive charge at pH 7.4. To analyze its function in the binding of IBMX, we mutated this position additionally to glutamine (Q, polar, neutral charge), or glutamic acid (E, acidic, negative charge) and compared it to alanine (A, nonpolar, neutral charge) and the wild type. It was found that the three mutants (R92A, R92Q and R92E) showed less sensitivity to IBMX compared to THIK-1 wild type, in particular the mutant THIK-1 Q92E showed the lowest sensitivity. These data strongly suggest that the linker region of K_{2P} channel plays an important role in the binding of IBMX. Furthermore, the charge and/or polarity of this domain greatly influence the direct binding of IBMX to the THIK-1 channel.

3.2 IBMX blocks THIK-2 also from extracellular side

We have shown that IBMX can directly bind the THIK-1 channel from extracellular side. Although THIK-2 shares a high sequence homology with THIK-1, human THIK-2 and rat THIK-2 channels are both described to be silent channels (Girard *et al.*,

2001; Rajan *et al.*, 2001). There are several potassium channels that seem to be silent, meaning non-functional in heterologous expression systems. However, under specific physiological conditions such channels may conduct currents, thus fulfilling yet undescribed functions. THIK-2 could be such a channel and any putative THIK-2 currents may be blocked by IBMX, analogue to THIK-1.

3.2.1 Trafficking mutants of THIK-2 give measurable currents and increase surface expression

When the THIK-2 wild type channel channel is expressed in CHO cells, no functional currents could be measured at high extracellular potassium solution (140 mM K^+, Figure 3.2.1B), just as reported.

THIK-2 channel shares high sequence identity to THIK-1. In rat, THIK-2 shows 58% sequence identity to THIK-1 which could produce robust currents in heterologous expression systems. Sequence alignment between THIK-2 and THIK-1 revealed that the THIK-2 channel has an additional amino acids sequence containing 19 amino acids from proline 6 (P6) to cysteine 24 (C24) while this sequence is missing in the THIK-1 channel (Figure 3.2.1A). A THIK-$2^{\Delta 6\text{-}24}$ mutant was used in which these additional amino acids were deleted. Intriguingly, THIK-$2^{\Delta 6\text{-}24}$ expressed in CHO cell could produce functional and robust potassium currents (Figure 3.2.1C). Under symmetrical 140 mM K^+ conditions, THIK-$2^{\Delta 6\text{-}24}$ currents showed slightly inward rectification. To further investigate the reason why THIK-$2^{\Delta 6\text{-}24}$ could produce functional currents, a THIK-2^{AAA} mutant was analyzed in which the amino acids 14 to 16 (RRR) were mutated to AAA. RXR is an arginine-based endoplasmic-reticulum retention/retrieval motif, which potentially prevents the wild type THIK-2 from reaching the plasma membrane (Michelsen *et al.*, 2005; Banfield, 2011). In a 140 mM K^+ condition, THIK-2^{AAA} could produce functional potassium currents. The currents showed slightly inward rectification just like the THIK-$2^{\Delta 6\text{-}24}$ construct under symmetrical conditions (Figure 3.2.1D). 1 mM quinidine was used as positive control to eliminate the leaking effect because at 140 mM K^+ bathsolution the membrane potential is nearly 0 mV.

CD74, also known as HLA class II histocompatibility antigen gamma chain or HLA DR antigens-associated invariant chain, contains an ER retention signal (MHRRRSR) which effectively retains the protein in the ER. In contrast, a retention signal mutant of CD74 (MHSSSSS) strongly express in cell membrane. The extracellular domain of CD74 can be easily detected, therefore it is often used as a reporter protein for surface expression, in particular if cytosolic domains of other proteins are 'transplanted' to CD74 in order to test their trafficking properties (Schutze *et al.*, 1994; Zuzarte *et al.*, 2009). When the N-terminus of wild type THIK-2 (RRR, red) was fused to CD74 and the chimera was expressed in COS-7 cells, the surface expression was as low as CD74 wild type (MHRRRSR). In contrast, when the mutated N-terminus of THIK-2 (AAA, blue) was fused to CD74, the surface expression increased more than threefold (Figure 3.2.1E). CD74 containing the N-terminal sequence of MHSSSSS was used as a negative control, giving the highest surface expression. This indicates that THIK-$2^{\Delta 6\text{-}24}$ and THIK-$2^{\Delta 6\text{-}24}$ induce measurable currents because the channels reach the cell surface. Therefore we refer to them as trafficking mutants. This part was performed by Dr. Vijayaram Renigunta (CD74 part).

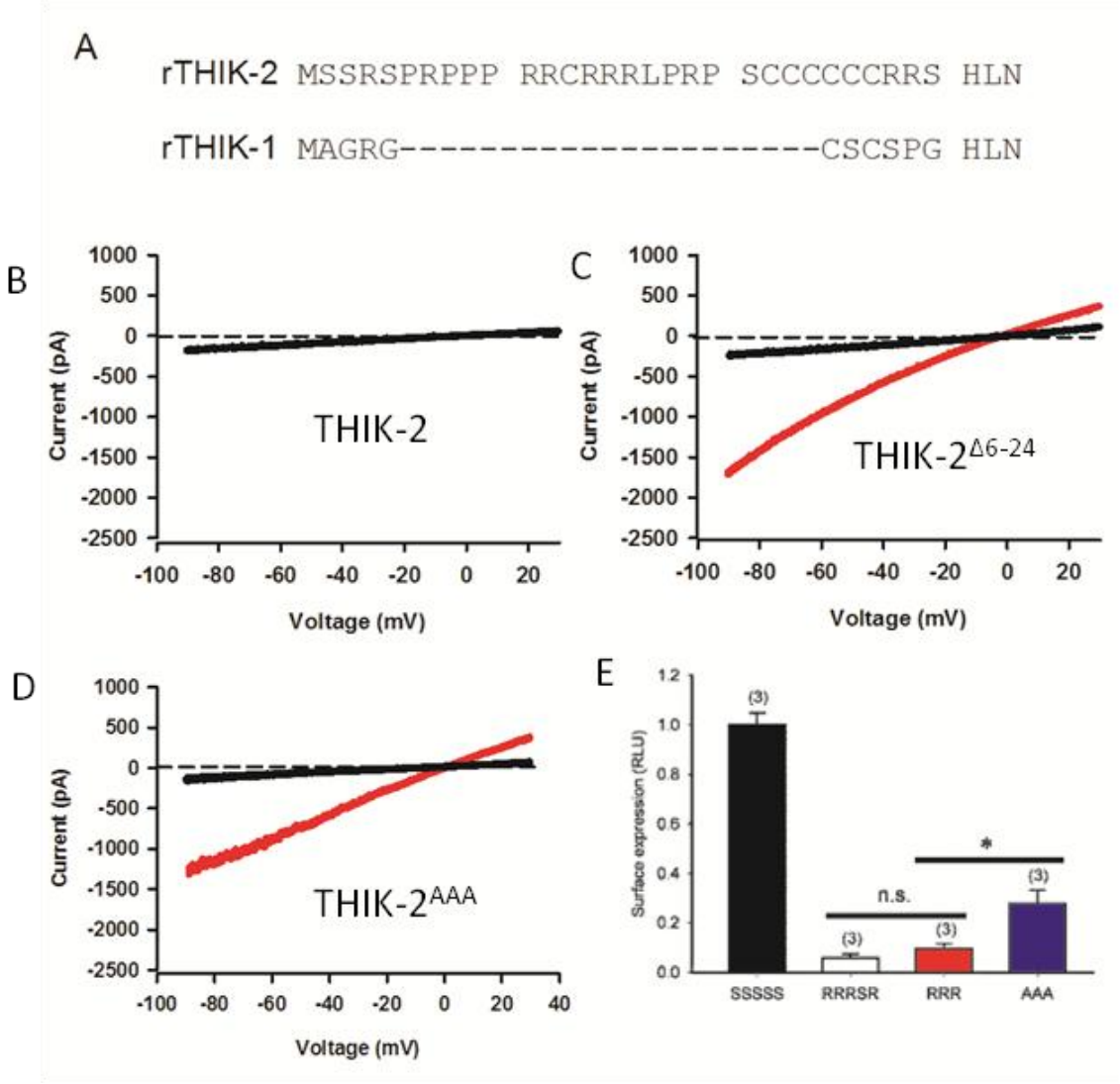

Figure 3.2.1 Trafficking mutants of THIK-2 give measurable currents and show increased surface expression. (A) The alignment of THIK-1 and THIK-2 amino acids sequence. (B) Typical current-voltage relationship of THIK-2 wild type expressed in CHO cell with 140 mM K^+ bathsolution. (C) Typical current-voltage relationships of THIK-$2^{\Delta 6\text{-}24}$ expressed in CHO cell with 140 mM K^+ bathsolution. The black line indicates the current-voltage relationship measured after application of 1 mM quinidine. (D) Typical current-voltage relationships of THIK-2^{AAA} expressed in CHO cell with 140 mM K^+ bathsolution. The black line indicates the current-voltage relationship measured after application of 1 mM quinidine. (E) The surface expression of CD74 containing the wild type N-terminus of THIK-2 (RRR) or the mutated N-terminus of THIK-2 (AAA) expressed in COS-7 cells. CD74 containing the N-terminus sequence of MHRRRSR was used as a positive control. CD74 containing the N-terminus sequence of MHSSSSS was used as a negative control. This experiment was performed by Dr. Vijayaram Renigunta.

3.2.2 Pore mutation increases THIK-2^{AAA} current

It was reported that TWIK-1 possesses a hydrophobic barrier deep within the inner pore and this hydrophobic barrier acts as the major barrier to ion conduction. Pore mutation at Leu 146 in the middle of the M2 helix increased the currents of TWIK-1 trafficking mutant (Chatelain *et al.*, 2012; Aryal *et al.*, 2014). At the corresponding position, the THIK-2 channel has an isoleucine residue (I158). Based on this, the THIK-2$^{AAA+I158G}$ double mutant was constructed. This construct produced a robust current that is higher compared to THIK-2^{AAA} construct (Figure 3.2.2A).

We found that all the mutants of THIK-2 (THIK-2^{AAA}, THIK-2$^{\Delta 6\text{-}24}$ and THIK-2$^{AAA+I158G}$) could produce potassium currents. Since 1 mM quinidine blocked the THIK-2 currents completely, the amplitude of the currents produced by the different mutants was calculated as the difference between the currents measured before and after application of the blocker quinidine:

We found that THIK-2$^{AAA+I158G}$ showed the largest currents and THIK-2^{AAA} showed the smallest. The current density of THIK-2^{AAA} was more than five times larger than that of THIK-2 wild type. The differences between thes constructs were all significant (Figure 3.2.2B). For the convenience of statistics we defined that 1 mM quinidine can completely block the currents of THIK-2 mutants to zero except THIK-2 wild type because its currents were too small to use quinidine.

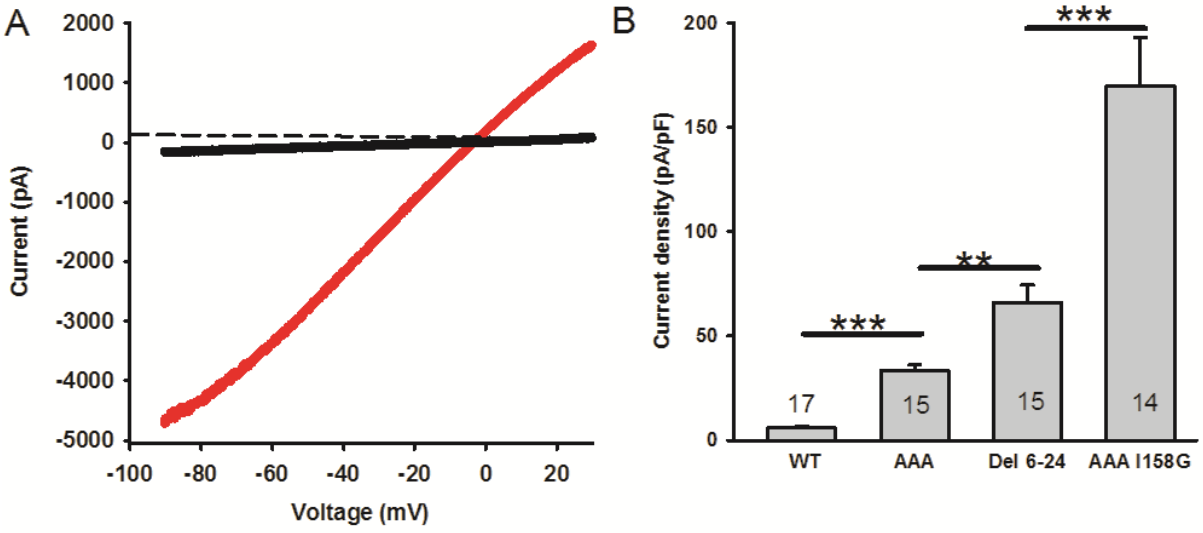

Figure 3.2.2 Pore mutation increased the currents of the trafficking mutant. (A) Typical current-voltage relationship of THIK-2$^{AAA+I158G}$. THIK-2$^{AAA+I158G}$ channel was expressed in CHO cells: the extracellular bath solution contained 140 mM K^+. The black line indicates the current-voltage relationship measured after application of 1 mM quinidine. (B) Statistics of the current density of THIK-2 and mutants measured in CHO cell at -80 mV. **, P <0.01; ***, P <0.001. The currents measured after application of 1 mM quinidine were defined as zero at -80 mV. The number of the measured cell is given in black; the error bars indicate SEM.

3.2.3 Effect of quinidine on THIK channel

In this study, we used quinidine to block THIK-2 mutants. All of the mutants of THIK-2 are sensitive to quinidine. The THIK-2$^{\Delta 6-24}$ mutant was used to calculate the IC_{50} value of quinidine. We used 50 µM, 100 µM and 250 µM quinidine to measure the dose response, while 1 mM quinidine was used to set 100% inhibition (zero currents) (Figure 3.2.3A). The IC_{50} of quinidine was about 100 µM in CHO cell. We also tested the sensitivity of THIK-1 channels to quinidine. This measurement was performed in oocytes because the current are more stable using this system. However, THIK-1 expressed in oocytes was less sensitive to quinidine compared to the THIK-2 mutants. The relative current of THIK-1 with 1 mM quinidine was only 0.43±0.01 (control = 1, Figure 3.2.3B).

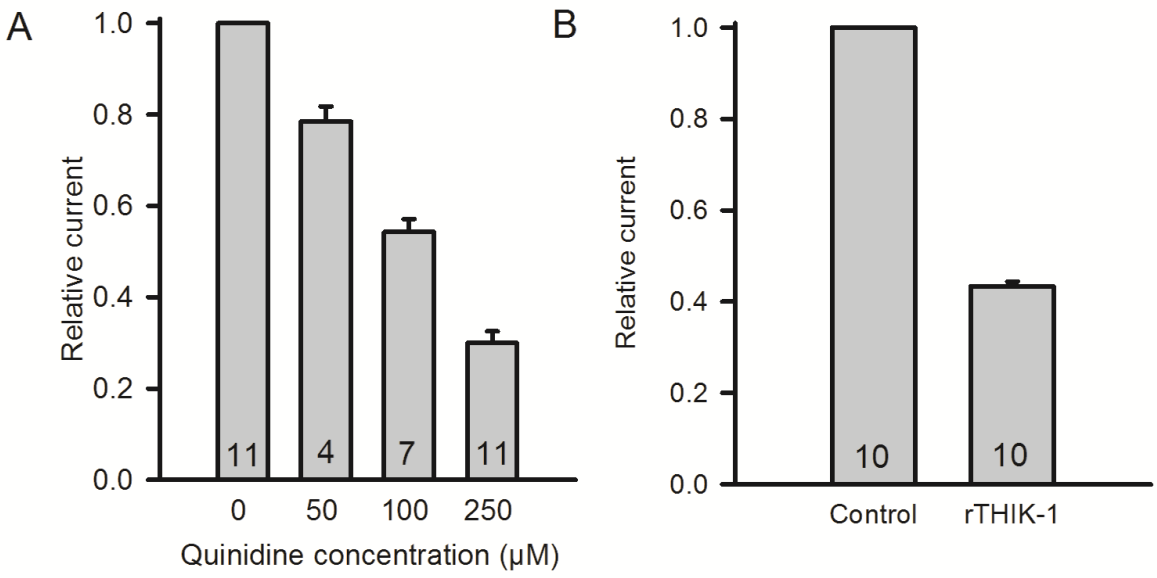

Figure 3.2.3 The relative current of THIK-2$^{\Delta 6\text{-}24}$ mutant and THIK-1 with quinidine. (A) Statistics of the effects of application of different concentrations of quinidine on the THIK-2$^{\Delta 6\text{-}24}$ mutant expressed in CHO cells. The currents of THIK-2$^{\Delta 6\text{-}24}$ were measured at -80 mV. 1 mM quinidine was used to set 100% inhibition (zero currents). (B) Statistics of the effects of 1 mM quinidine on THIK-1 wild type expressed in oocytes. The currents of THIK-1 channel were measured at 0 mV. The number of the measured cell is given in black; the error bars indicate SEM.

3.2.4 Block of THIK-2 by IBMX

We found that IBMX could block the THIK-1 channel from the extracellular side. The THIK-2 channel shares a high sequence identity with THIK-1, in particular the trafficking mutant THIK-2$^{\Delta 6\text{-}24}$ because the deleted amino acids 6-24 are missing in THIK-1. As the THIK-2$^{\Delta 6\text{-}24}$ mutants give measurable currents, the effect of IBMX on THIK-2$^{\Delta 6\text{-}24}$ was tested.

Our results suggest that IBMX could indeed block THIK-2$^{\Delta 6\text{-}24}$ channel currents (Figure 3.2.4). Outward and inward currents of THIK-2$^{\Delta 6\text{-}24}$ were all affected by IBMX, comparable to the effect on THIK-1 channel. The inhibition kinetics of IBMX on the THIK-2$^{\Delta 6\text{-}24}$ channel was, like for the THIK-1 channel, very fast and the inhibition could be reversed by washout (Figure 3.2.4B). Compared to THIK-1, THIK-2$^{\Delta 6\text{-}24}$ was less sensitive to IBMX, the currents of THIK-2$^{\Delta 6\text{-}24}$ were inhibited only by ~39% at an IBMX concentration of 1 mM IBMX (n=8) (Figure 3.2.4C).

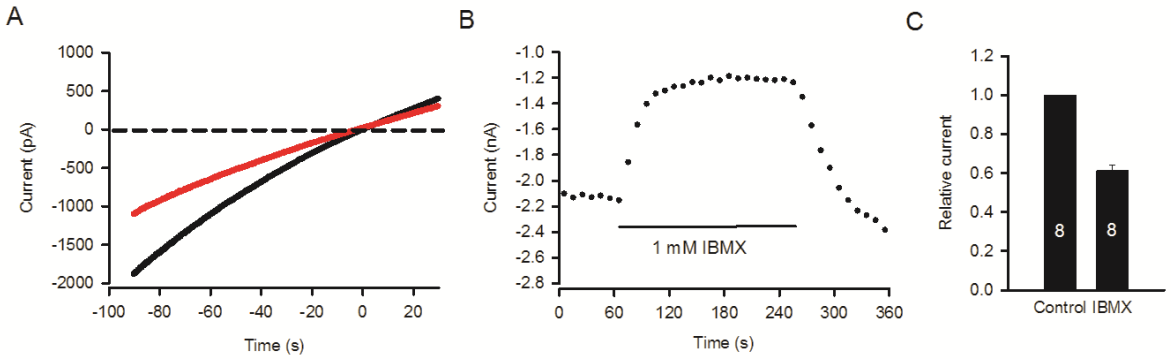

Figure 3.2.5 The effect of IBMX on the THIK-2$^{\Delta 6\text{-}24}$ mutant expressed in CHO cell. A. Current-voltage relationship of THIK-2$^{\Delta 6\text{-}24}$ mutant with or without application of 1 mM IBMX at holding

potential of -80 mV. Red, with 1mM IBMX. Black, without IBMX. (B) Time course of the current change recorded during application and washout of 1 mM IBMX on THIK-$2^{\Delta 6\text{-}24}$ construct in CHO cells at -80 mV. (C) Statistics of the effect of 1 mM IBMX on THIK-$2^{\Delta 6\text{-}24}$ construct. The number of cells studied is given in white; the error bars indicate SEM.

3.2.5 The effect of cAMP on THIK-$2^{\Delta 6\text{-}24}$ channel

In previous sections, the common function of IBMX as a PKA activator was discussed. We have shown that the inhibition of THIK-1 by IBMX is not due to increased intracellular cAMP levels. As the trafficking mutant THIK-$2^{\Delta 6\text{-}24}$ is also inhibited by IBMX, it should be analyzed, if this effect is comparably not mediated by cAMP. In an analogue experiment, cAMP was applied into the patch pipette and the patch membrane was ruptured by a negative pressure. The currents were subsequently measured at holding potential of -80 mV with 140 mM K^+ bath solution. After rupturing the patch membrane an inward current was measured. This current was constant throughout the measurement while cAMP diffused into the CHO cell cytosol through the patch pipette (Figure 3.2.5). As a positive control, TREK-1c expressed in CHO cells showed a rapid decay of the outward current at holding potential of 0 mV (Figure 3.1.3C).

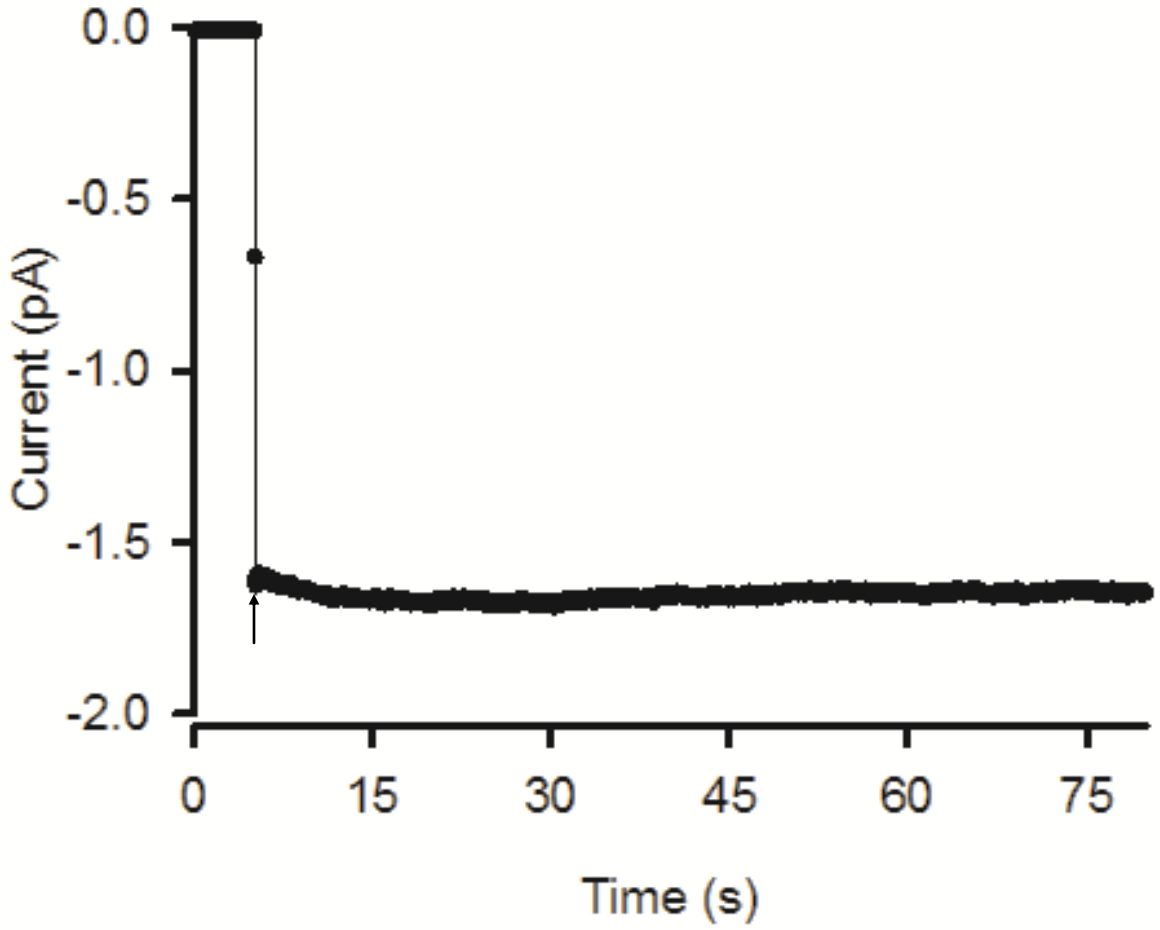

Figure 3.2.5 The effect of 100 µM intracellular cAMP applied through patch pipette on THIK-2 mutant channel expressed in CHO cells at holding potential of -80 mV. A cell-attached patch was formed and then ruptured by a negative pressure at the red arrow.

3.2.6 IBMX blocks THIK-2$^{\Delta 6\text{-}24}$ currents from extracellular side

We have shown that IBMX blocks THIK-1 channels directly from the extracellular side, independent of the cAMP pathway. The trafficking mutant THIK-2$^{\Delta 6\text{-}24}$ similarly does not react on increased intracellular cAMP levels, suggesting the same inhibition mechanism of IBMX. To prove that IBMX also blocks THIK-2 channels only from the extracellular side IBMX was applied intracellularly or via the bath solution in the same way as it was done for THIK-1. Briefly, 1 mM IBMX was applied into the patch pipette and after formation of the Gigaseal the membrane was ruptured and the inward

current was measured at holding potential of -80 mV. After 60 seconds, 1 mM IBMX was washed in via the bath solution and washed out after additional 120 seconds. The results were comparable to those obtained from THIK-1: IBMX applied intracellularly did not affect the currents of the THIK-$2^{\Delta 6\text{-}24}$ mutant (Figure 3.2.6A) but IBMX in the bath solution rapidly and reversibly inhibited the currents (Figure 3.2.6B). The double (trafficking and pore) mutant THIK-$2^{AAA+I158G}$ significantly increased currents compared to THIK-2^{AAA} and THIK-$2^{\Delta 6\text{-}24}$ (Figure 3.2.2). To test whether IBMX also blocks the pore mutant or not, we applied 1 mM IBMX on THIK-$2^{AAA+I158G}$ channels expressed in CHO cell in a 140 mM K^+ bath solution. Interestingly, IBMX could not block the currents of the THIK-$2^{AAA+I158G}$ channel (Figure 3.2.6D). Next, the pore mutant of THIK-1 at the corresponding position (THIK-1^{I139G}) was also analyzed in oocytes. The application of 500 µM IBMX did not alter the THIK-1^{I139G} currents (Figure 3.2.6 C, E). Those results indicate that this pore mutation interfere with the inhibitory effects of IBMX.

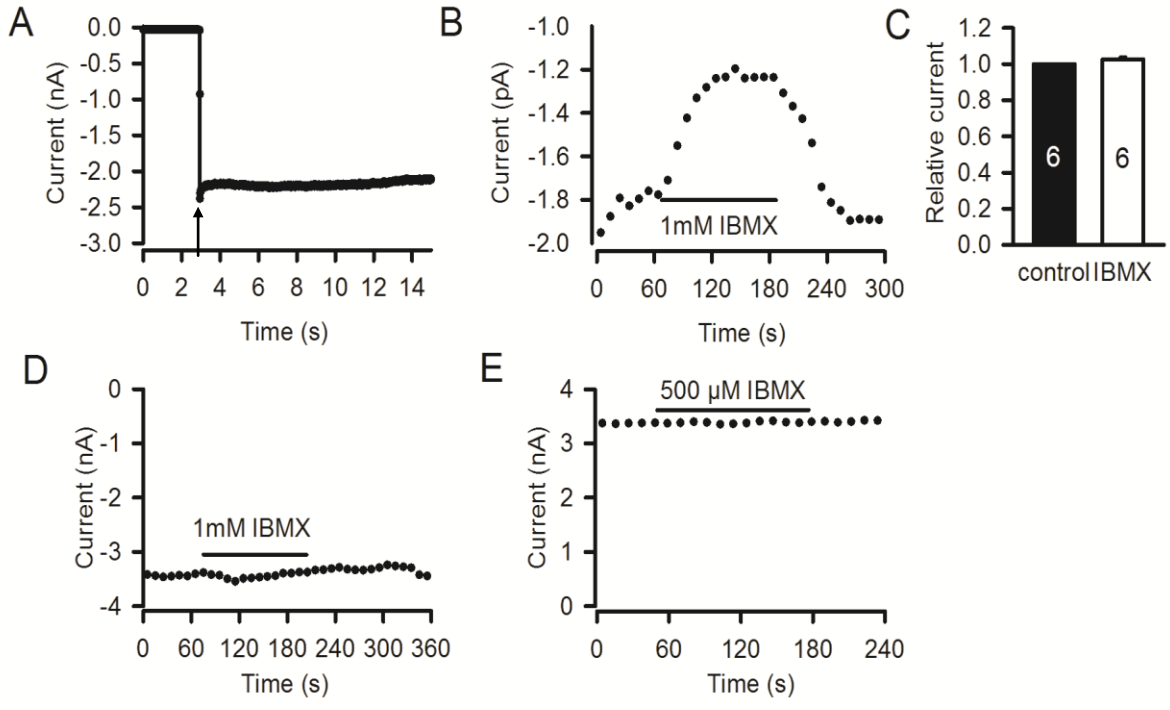

Figure 3.2.6 IBMX blocks THIK-$2^{\Delta 6\text{-}24}$ from extracellular side but not the pore mutants THIK-$2^{AAA+I158G}$ and THIK-1^{I139G}. (A) The effect of application of 1 mM intracellular IBMX via patch pipette in a CHO cell transfected with THIK-$2^{\Delta 6\text{-}24}$. (B) The effect of application of 1 mM

intracellular IBMX via patch pipette and 1 mM extracellular via bath solution in representative CHO cell transfected with THIK-$2^{\Delta 6\text{-}24}$. The results shown in Figure A and B were measured in the same cell. (C) Statistics of the effects of 500 µM IBMX on THIK-1^{I139G} currents. Relative current means currents after application of IBMX in comparison to before the application of IBMX by TEVC at potential of 0 mV. The number of the measured cell is given in black or white. (D) Application of 500 µM IBMX on THIK-$2^{AAA+I158G}$ channels expressed in CHO cell by whole cell recording at potential of -80 mV. (E) Application of 500 µM IBMX on THIK-1^{I139G} channels expressed in oocytes at membrane potential of 0 mV. The extracellular potassium concentration was 2 mM.

4 Discussion

4.1 IBMX blocks THIK-1 channel from extracellular side

3-siobutyl-1-methyl-xanthine (IBMX) is commonly used as inhibitor of the enzyme phosphodiesterase, resulting in an increase of cAMP levels (Essayan, 2001). IBMX is therefore used to activate cAMP pathways including the PKA pathway and the Epac pathway. For example, IBMX was used to analyze the properties of TREK-1 channels (Fink *et al.*, 1996), T-type Ca^{2+} channels (Gautam *et al.*, 2007) and ATP-sensitive K^+ (K_{ATP}) channels (Morales *et al.*, 2004; Herbst *et al.*, 2011). However, the possibility that IBMX may induce its properties independent from cAMP was not considered.

To study the signal transduction of THIK-1 channel we used IBMX to increase cAMP levels. We found that IBMX can significantly block the outward and inward currents of THIK-1 channels in CHO cell and in oocytes (Figure 3.1.1). The block of THIK-1 currents IBMX was nearly as fast as the change of bath solution, hence much faster than it would be expected if cAMP signaling would be involved. However the dissociation of IBMX effect was significantly slower than the solution change (Figure 3.1.1C).

To further study the role of the cAMP pathway in THIK-1 channel regulation, H89 and forskolin were used. Basically, H89 is used as PKA inhibitor which can block PKA signaling pathway by acting as competitive antagonists at the ATP site of the PKA catalytic subunits (Murray, 2008). Surprisingly, the application of high concentrations of H89 did not affect the currents of THIK-1. In presence of H89, IBMX still could inhibit THIK-1, but there is no additional effect (Figure 3.1.2A-C). Forskolin is a labdane diterpene which can activate the enzyme adenylyl cyclase, resulting in an increase of intracellular cAMP level. Generally, people use IBMX and forskolin together to fully activate the cAMP pathway because forskolin activates adenylyl cyclase, and IBMX inhibits phosphodiesterase (Kang *et al.*, 2007; Dixon *et al.*, 2011; Cuppoletti *et al.*, 2014). However, even very high concentration of forskolin did not affect the THIK-1 currents. Another K_{2P} channel, human TREK-1c, was used as a positive control for active cAMP signaling because the channel is inactivated after phosphorylation by PKA (Terrenoire *et al.*, 2001; Rinné *et al.*, 2013). There was

nearly complete and reversible inhibition after application of 20 µM forskolin, indicating that forskolin definitely acts as an inducer of cAMP signaling (Figure 3.1.2).

All the above results look like indistinct and paradoxical. To further study the mechanism of inhibition of THIK-1 by IBMX we applied cAMP directly into the cytosol of CHO cells transfected with THIK-1 by introduce 100 µM cAMP into the patch pipette. After formation of the gigaseal, the patch membrane was ruptured by applying negative pressure and the currents were measured at holding potential of 0 mV. We found that cAMP did not alter the currents of THIK-1. TREK-1c wild type was used as a positive control because it is regulated by IBMX and forskolin though cAMP pathway, more precisely by phosphorylation by PKA. The mutant TREK-1c S344A was used as a negative control as it lacks the PKA phosphorylation site (Gonzalez *et al.*, 2012). As expected, cAMP can almost fully inhibit the currents of TREK-1c, but not that of the TREK-1 S344A mutant (Figure 3.1.3).

All the above mentioned observations suggested one possibility: IBMX blocks the THIK-1 channel though directly binding the channel, regulating its activity independent from the cAMP signal pathway.

To support this hypothesis, IBMX and TPA were applied either from the intra- or the extracellular side. When 1 mM IBMX was introduced into the patch pipette the currents of THIK-1 were unaffected; this indicates that intracellular IBMX does not inhibit the channel. When the same cell was subsequently superfused with 1 mM IBMX the currents of the channel were rapidly blocked by IBMX; this indicates that only extracellular IBMX can inhibit THIK-1 currents. As a positive control, TREK-1c was used because it is phosphorylated after application of IBMX (Fink *et al.*, 1996; Maingret *et al.*, 2000). TPA was used as a positive control. TPA blocks potassium channels directly from the intracellular side (Sanchez and Blatz, 1995; Oliver *et al.*, 1998; Piechotta *et al.*, 2011). In our experiments, TREK-1 currents could be blocked by intracellular IBMX or TPA. However, THIK-1 currents were blocked by extracellular IBMX or intracellular TPA, but not intracellular IBMX (Figure 3.1.4). In conclusion, all the results mentioned above support the idea that the inhibition of IBMX on THIK-1 currents is mediated by a direct block from the extracellular side, but not via signal transduction.

Further research suggested that the inhibition of IBMX to THIK-1 was specific because IBMX could not inhibit TRAAK currents (Figure 3.1.4D), another K_{2P} channel that is not regulated by PKA and PKC kinases (Fink *et al.*, 1998). The IC_{50} value of IBMX inhibiting THIK-1 expressed in CHO cell was about 152 µM. However, in oocytes the sensitivity was lower than in CHO cell, for example the relative block of 500 µM IBMX in oocytes was about 60%, whereas it was about 75% in CHO cells (Figure 3.1.4F-H). So far, the reason for this difference remains unclear, although it has been observed with many drugs (Staudacher K et al. 2011; Putzke et al. 2007).

To identify the IBMX binding site on THIK-1, amino acids of the helical cap region of the channel were mutated one by one. The effects of IBMX to THIK-1wild type and generated mutants were measured and compared in oocytes. Most of the mutants showed a residual current (I_{IBMX}/I_0) of about 0.4 of control after application of 500 µM IBMX, similar to THIK-1 wild type except the mutants R92A, K50A and G85A, indicating potential IBMX binding sites. Alignment of the helical cap region of the K2P channels among TWIK-1, TRAAK, TREK-2, THIK-1 and THIK-2 revealed that the cap helices 1 and 2 are highly conserved in these channels, except for the linker region between cap helix 2 and the pore helix (C2-P1 linker, Figure 3.1.5). Part of the linker region was found to be disordered in K_{2P} family and is also not visible in the crystal structure. Interestingly, the arginine residue at position 92, which showed the largest decrease of the effects of IBMX in the mutants scan, is at the end of the unstructured region, three residues proximal to the pore helix.

To further study the character of the C2-P1 linker region of K_{2P} channel, a THIK-1/TREK-1 chimera was used in which the linker region of THIK-1 was replaced by TREK-1's (Figure 3.1.5). We found that the chimera shows significantly less sensitivity to different concentrations of IBMX. Additionally, in THIK-1 wild type arginine 92 was mutated to glutamine (Q, polar and neutral charge), glutamic acid (E, acid polarity and negative charge) or alanine (A, nonpolar, neutral charge). We found that all three mutants (R92A, R92Q and R92E) show less sensitivity to IBMX compared to THIK-1 wild type. Mutant THIK-1 Q92E shows the lowest sensitivity to IBMX of the three mutants. These data indicate that the linker region of K_{2P} channel plays an important role in the binding of IBMX, in particular the charge or polarity of the region influences the direct binding of IBMX to THIK-1 channel.

4.2 Breaking the silence of THIK-2 channel

K_{2P} family has fifteen members. However, five of them, THIK-2, TWIK-1, TWIK-2, TASK-5 and KNCK7 were described as silent channel because no or only very weak inwardly rectifying currents could be measured in heterologous expression systems just like description in section 1.3. Now the reasons of TWIK-1 silence were reported: It contains a diisoleucine-based motif in its cytoplasmic C-terminus, which mediates rapid endocytosis. Diisoleucine-based motifs are classical signals for endocytosis, and 'D/E(XXXL)L/I' is their consensus sequence (Bonifacino and Traub, 2003). After mutation of the double isoleucine to alanine, TWIK-1 produced robust potassium currents. Further research showed that mutation of double isoleucine will stabilize TWIK-1 channel at the plasma membrane, stopping the endocytosis (Feliciangeli *et al.*, 2010). Another potential reason why TWIK-1 currents are small is that TWIK-1 possesses a hydrophobic barrier deep within the inner pore and this hydrophobic barrier acts as a major barrier to ion conduction (Aryal *et al.*, 2014). For the other four silent K_{2P} channels, the mechanisms of keeping silence are still not clear.

THIK-2 channel has a high sequence identity with THIK-1 (59% in the rat) (Rajan *et al.*, 2001). However the physiological properties of the two channels are quite different. THIK-1 channel can be functionally and heterologously expressed, whereas heterologous expression of THIK-2 could not produce any measureable current although THIK-2 channel was found in cell membrane (Rajan *et al.*, 2001; Lazarenko *et al.*, 2010). However, the targeting of THIK-2 to the cell membrane was significantly less than that of THIK-1. Until now all the attempts were unsuccessful to break the silence of THIK-2 channel. In this dissertation we transfected THIK-2 into CHO cell and got the same result (Figure 3.2.1B).

Sequence alignment between rTHIK-2 and rTHIK-1 revealed that THIK-2 has an additional N-terminal domain (amino acids position from 6 to 24) which is missing in THIK-1 channel. After deletion of this domain, THIK-$2^{\Delta 6\text{-}24}$ mutant produced robust and functional inwardly rectifying currents (with a high concentration of potassium in the bath solution). This construct is highly sensitive to quinidine. 1 mM quinidine could almost completely inhibit the currents. The IC_{50} of quinidine to THIK-$2^{\Delta 6\text{-}24}$ in CHO cell was about 100 µM (Figure3.2.1 and 3-2-2). Because we used a high

potassium bath solution and in this condition there is no membrane potential, we used quinidine as positive control and found that 1 mM completely inhibited the currents. However THIK-1 channel is not so sensitive to. quinidine. With 1 mM quinidine, the inhibition was less than 60% (Figure 3.2.3).

To further investigate the reason why THIK-2$^{\Delta 6\text{-}24}$ could produce functional currents, we constructed THIK-2^{AAA} channel by mutating amino acids of 14-16 RRR to AAA because RXR is an endoplasmic reticulum retention/retrieval signal (Michelsen *et al.*, 2005; Banfield, 2011). THIK-2^{AAA} produced functional potassium currents and was sensitive to quinidine although the currents were smaller than in the THIK-2$^{\Delta 6\text{-}24}$ construct (Figure 3.2.1 and 3.2.2). The currents of THIK-2^{AAA} showed slight inward rectification like the THIK-2$^{\Delta 6\text{-}24}$ construct in 140 mM K^+ bath solution. So we speculate that the endoplasmic reticulum retention/retrieval signal RRR stopped the THIK-2 export to cell membrane, leading to the silence of the channel. The retention signal in the N-terminus of THIK-2 could also be transplanted to the reporter protein CD74 (Figure 3.2.2 and Renigunta et al., 2014). All those data suggest that ER retention signal RRR stop THIK-2 to transfer to cell membrane, leading to no current can be measured.

It was reported that TWIK-1 possesses a hydrophobic barrier deep within the inner pore and this hydrophobic barrier acts as a major barrier to ion conduction. When polar substitutions were made at Leu146, very large whole cell currents could be recorded (Aryal *et al.*, 2014). Sequence alignment between THIK-2 and TWIK-1 revealed that isoleucine158 of THIK-2 is the corresponding position of TWIK-1 Leu146. We constructed THIK-2$^{AAA+I158G}$ mutant by mutating an isoleucine residue to glycine and the ER retention signal RRR to AAA, because all K_{2P} channels have a glycine residue at the position except TWIK (Leu) and THIK channel (Iso) (Ben-Abu *et al.*, 2009). THIK-2$^{AAA+I158G}$ construct produced robust and bigger currents than THIK-2^{AAA} or THIK-2$^{\Delta 6\text{-}24}$. The current density of THIK-2$^{AAA+I158G}$ construct was almost sixfold bigger than THIK-2^{AAA} construct (Figure 3.2.2B). All the three THIK-2 mutants revealed a slight inward rectification. Those above data suggest that we have broken the silence of THIK-2 channel. The reason of THIK-2 silence has two mechanisms: 1, Trafficking reason. THIK-2 possesses an ER retention signal, leading to less THIK-2

molecules arrive at the cell membrane. 2, Gating reason. THIK-2 may possess a hydrophobic barrier deep within the inner pore which stops ion conduction.

Recently another group also reported the similar data about breaking the silence of human THIK-2 channels. We all used the similar method and deleted similar sequence of THIK-2, and then got the similar IV curve of THIK-2 (Chatelain *et al.*, 2013).

4.3 IBMX blocks THIK-2 channels from extracellular side

In previous sections we proved that IBMX can directly block THIK-1 channel from extracellular side and measured functional expressed THIK-2 currents. To identify the effect of IBMX on THIK-2 channel, we applied 1 mM IBMX on THIK-$2^{\Delta 6\text{-}24}$ mutant. We found that IBMX can inhibit THIK-$2^{\Delta 6\text{-}24}$ currents and the inhibition is reversible. However the inhibition of 1 mM IBMX on THIK-2 was less than 40% (Figure 3.2.4). To exclude cAMP pathway from the inhibition, we applied 100 µM cAMP into the pipette solution and measured the currents before rupturing the patch membrane. Just like in THIK-1 channel, cAMP did not affect the currents of THIK-2, indicating that maybe IBMX blocks THIK-$2^{\Delta 6\text{-}24}$ also from extracellular side (Figure 3.2.5). To verify this hypothesis we used the same method to prove the hypothesis which we have discussed in the previous sections (Discussion 4.1). Those data reveal that IBMX blocks THIK-2 $^{\Delta 6\text{-}24}$ channel also from extracellular side.

Because the reasons of keeping THIK-2 channel silence have two aspects: Trafficking and gating. We also applied 1 mM IBMX in THIK-$2^{AAA+I158G}$ mutant. Surprisingly we found that IBMX could not inhibit the currents of THIK-$2^{AAA+I158G}$ at all. At the corresponding position the currents of THIK-1^{I139G} expressed were also not inhibited by IBMX (Figure 3.2.6). Unfortunately we still don't understand the reason.

At THIK-1 channel we study the binding site of IBMX in the linker region, however we paused this research in THIK-2 because of time limitation.

4.4 The significance of our work

There were, are and will be a lot of researchers who use IBMX as a tool to study cell biology. Of course IBMX is also very convenient and useful chemical to study cell biology. Our works give people another aspect that we should take into consideration: IBMX is also an ion channel blocker. On the other hand our discovery may be useful for detecting endogenous THIK-1 channels by blocking them with extracellular IBMX.

The protein expression profile is different between THIK-1 and THIK-2. For example, THIK-2 was found in the anterodorsal and anteroventral nuclei, thalamic ventral posterior nuclei cortex, hippocampus, oculomotor nuclei and the cerebellum, where THIK-1 was not expressed (Rajan *et al.*, 2001). How does the THK-2 act as a functional potassium channel? We think there may be two ways. (1) THIK-2 acts functionally alone or with other protein, channel such as THIK-1 to regulate resting membrane potential and cell excitability in neurons. Recently is was reported that THIK-1 and THIK-2 can assemble and form an active channel (Blin *et al.*, 2014). (2) THIK-2 channel possesses an ER retention signal and is mainly localized to the ER region. Thus, THIK-2 may play an important role in the ER.

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Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren

in Marburg: Daut und Renigunta.

in Shanghai: Liao und Ge.

In Guangzhou: Zeng und Su.

Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel “An extracellular drug binding site of the potassium channels THIK-1 and THIK-2 ” im Institut für Physiologie und Pathophysiologie der Philipps-Universität Marburg unter Leitung von Prof. Dr. Dr. Jürgen Daut ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Marburg, den 07. 04. 2015

(Xinle Zou)